Interaction of Fe(III)-reducing bacteria with heavy metals in contaminated soils

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Introduction

Dissimilatory Fe(III)-reduction

Iron is the fourth most abundant element on earth. It is ubiquitous in the hydrosphere, pedoshpere, lithosphere, biosphere and atmosphere (Cornell and Schwertmann 2003). Iron oxides are among the most abundant minerals in soils and undergo various interactions with other soil components (Canfield et al. 2005, Kappler and Straub 2005). Iron occurs in two oxidation states, Fe(II) and Fe(III), that easily transform into one another under conditions commonly encountered in nature (Nealson and Saffarini 1994). This convertibility makes iron an important component of proteins involved in electron transfers (Madigan et al. 2003, Canfield et al. 2005) and makes iron an essential trace element for the majority of organisms (Kappler and Straub 2005, Madigan et al. 2003). Also, reduction or oxidation of iron in dissimilatory pathways can be used by microorganisms to gain energy for growth and enables a biological cycling of iron between its oxidized and reduced form (Kappler and Straub 2005).

In contrast to assimilatory Fe(III)-reduction, dissimilatory Fe(III)-reducing microorganisms conserve energy by transferring electrons from the respiratory chain to Fe(III) as a terminal electron acceptor and reducing it to Fe(II) (Lovley and Phillips 1988, Lovley 2006). Fe(III)-reducing microorganisms play a major role in carbon turnover in freshwater and marine sediments, submerged soils, and aquifers (Nealson and Saffarini 1994, Canfield et al. 2005, Lovley 2006). The significance of Fe(III)-reduction is enhanced by the possible reoxidation of Fe(II) by chemical or biological processes, leading to a cycling of iron molecules, where each can be used multiple times as electron acceptor (Lovley 2006). Electron donors for Fe(III)-reduction are mainly short chain organic acids like acetate, originating from the fermentative break down of complex organic matter, but also hydrogen, aromatic compounds, and long-chain fatty acids (Coates et al. 1996, Coates et al. 1999, Lovley et al. 2004). At low pH, elemental sulfur can also serve as electron donor (Das et al. 1992, Pronk et al. 1992).

Several microorganisms are able to reduce Fe(III) but do not conserve energy from this process. Some fermentative microorganisms, like *Bacillus*, *Clostridium*, and *Pseudomonas* species (*Firmicutes* and γ-*Proteobacteria*, respectively) are able to partially (less than 5 %) transfer reducing equivalents to Fe(III). Also, many sulfate-reducing microorganisms can reduce Fe(III), but only one has been suggested to gain energy for growth from this process (Tebo and Obraztsova 1998, Lovley 2006).

Electron acceptors used by Fe(III)-reducing microorganisms

The reduction of different electron acceptors proceeds from the highest energy yield to the lowest. The higher the redox potential of an electron acceptor, the more energy can be conserved by its reduction. The sequence of declining redox potentials usually begins with the reduction of oxygen to water (O_2/H_2O), followed by nitrate to nitrite (NO_3^-/NO_2^-), manganese(IV) to manganese(II) (Mn(VI)/Mn(II)), ferric iron to ferrous iron (Fe(III)/Fe(II)), sulfate to sulfide (SO_4^{2-}/S_2^-), and carbon dioxide to methane (CO_2/CH_4) (Nealson and Saffarini, 1994). Therefore, Fe(III)-reduction is usually found in anoxic subsurface environments. Due to the small spatial heterogeneity of soils and sediments, redox processes often overlap in space and time (Alewell et al. 2006). As Fe(III)-reducing microorganisms can reduce a variety of Fe(III)-forms, the reduction potential of Fe(III) depends strongly on the speciation of the oxidized and reduced Fe(III)-species, ranging from 0.77 V for dissolved iron at pH 0 to 0.00 V for poorly crystalline Fe(III) oxide at neutral pH, and down to -0.30 V for crystalline Fe(III) oxides (Canfield et al. 2005, Thamdrup 2000). Sufficient amounts of dissolved Fe(III) are only available at acidic pH. Under pH-neutral conditions Fe(III) is highly insoluble and mostly present as stable solid phases. Here, poorly crystalline hydrous ferric oxide is the most favorable form of particulate Fe(III) for Fe(III)-reduction (Canfield et al. 2005). Also structural Fe(III) in clay minerals can be reduced, while the environmental relevance of crystalline Fe(III)-oxides is questionable (Kostka et al. 1999, Lovley et al. 2004).

The particulate form of Fe(III) at most environmental conditions raises a problem for Fe(III)-reducing microorganisms in accessing an insoluble electron acceptor. Fe(III)-reducing microorganisms have developed several strategies to access solid phase iron. *Geobacter* transfers electrons directly to the Fe(III)-mineral via electrically conductive pili structures (nanowires) (Reguera et al. 2005). *Shewanella* uses outer membrane cytochromes to catalyze Fe(III)-reduction through direct contact with the Fe(III)-mineral (Heidelberg et al. 2002, Lower et al. 2009), and the production of nanowires has also been described (Gorby et al. 2006). Fe(III) might also be solubilized by chelators and become available to the cell as it was suggested for *Geothrix* (Nevin and Lovley 2002). Instead of directly reducing Fe(III), several Fe(III)-reducing microorganisms transfer electrons to an external electron shuttle such as organic substances like chinones or humic acids that might be present in the environment or produced by the microorganisms themselves (Nevin & Lovley 2000, Lovley et al. 2004). These electron shuttles then carry the electron to the solid phase Fe(III) and regenerate by transferring the electron to Fe(III). Most FeRP also reduce other metals and metalloids, like

As(V), Co(III), Cr(VI), Mn(IV), Tc(VII), and U(VI), and other non-metallic electron acceptors, such as oxygen, nitrate, sulfur, fumarate, humic substances, chlorinated compounds, and electrodes as alternative electron acceptors (Lovley et al. 2004).

Phylogenetic affiliation of Fe(III)-reducing microorganisms

Dissimilatory Fe(III)-reduction has been proposed as one of the first forms of microbial metabolism (Vargas et al. 1998). Abiotic photochemical generation of Fe(III) and H_2 on a hot Fe(II)-rich, early earth would have provided an electron acceptor and electron donor, respectively (Cairns-Smith et al. 1992, Lovley 2004). All microorganisms that are most closely related with the last common ancestor of extant microorganisms are Fe(III)-reducers (Lovley, 2006). The ability to reduce Fe(III) is widespread among numerous phylogenetic and physiologic groups within both the *Eubacteria* and *Archaea*. In the *Eubacteria,* Fe(III)-reducing bacteria (FeRB) can be found within the α-, β-, γ-, δ-, and ε-*Proteobacteria*, *Acidobacteria*, *Actinobacteria*, *Deferribacteres*, *Thermotogales*, *Thermodesulfobacteriales*, the *Deinococcus-Thermus* group, and the *Firmicutes*. Among them are thermophilic organisms like *Bacillus infernus*, *Deferribacter thermophilus*, and *Thermotoga maritima*. Neutro- and mesophilic species, like *Shewanella* species and members of the *Geobacteraceae* (γ- and δ-*Proteobacteria,* respectively) especially have been studied intensively. Within the *Geobacteraceae* the genus *Geobacter* has received a great deal of interest. This genus is widely distributed in freshwater soils and sediments while another *Geobacteraceae* genus, *Desulfuromonas*, has primarily been cultured from marine environments (Lovley 2006). *Geobacteraceae* seem to be important Fe(III)-reducers both in culture and *in situ*, while the relevance of *Shewanella* for environmental Fe(III)-reduction remains questionable (Lovley et al. 2004, Canfield et al. 2005, Lovley 2006). Although the redox potential of the Fe(III)/Fe(II) redox couple is high at acidic conditions, making Fe(III)-reduction a favorable process, little is known about acidophilic FeRB, like *Acidiphilium* species (α- *Proteobacteria*). For *Acidiphilium cryptum* JF-5 the genome sequence is available (http://www.ncbi.nlm.nih.gov/nuccore/CP000697), making this strain an ideal model organism to study Fe-reduction under acidic conditions.

Due to their broad phylogenetic diversity, the identification of FeRB communities is difficult. Unlike sulfate-reducing prokaryotes, which have a common functional gene, *dsrAB*, which can be used for phylogenetic analyses of sulfate-reducing microbial populations (Wagner et al. 2005), no such common functional gene is known for FeRB. Consequently,

FeRB communities have to be studied by other methods. FeRB can be identified by cultivation and enrichment techniques; however, this approach excludes all FeRB that are not able to grow under the chosen culture conditions. Selected groups of FeRB, like *Geobacteraceae*, may be examined by specific nucleic acid probes or primer sets, but this method can only detect known FeRB. One method to identify the active members of an Fe(III)-reducing microbial population is by stable isotope probing (SIP). The microbial community can be linked with this method to a specific process, like Fe(III)-reduction (Radajewski et al. 2003, Friedrich 2006). An isotopically labeled carbon source is consumed by the microorganisms, which are actively growing during Fe(III)-reduction, and the label is incorporated into the cell material. FeRB can then identified by analysis of the isotopically labeled DNA. Another method for the identification of microorganisms is Raman spectroscopy (Schmitt and Popp 2006). With this method, specific fingerprint-like spectra containing information about cell components, like nucleic acids, proteins, lipids, or polysaccharides, can be obtained. Microbial species or strains can then be identified by comparing these spectra with a database. This method has been applied for identification of microorganisms in clean room environments and lactic acid bacteria from yoghurt where only few microbial species are present (Rösch et al. 2005 a, Gaus et al. 2006). With an appropriate spectra database this method also has the potential to be used for identification of more complex microbial communities, like FeRB.

Heavy metals and their environmental relevance

There is no official definition of the term “heavy metal”, although this term is usually applied to elements with a density > 5 g cm^{-3} (Duffus 2002). Although many metals are important micronutrients, they are toxic for organisms, including humans, at higher concentrations and can enter the food chain (Bruins et al. 2000, Giri et al. 1980, Sharma and Agrawal 2005). Also, the metalloid As is toxic and poses a health risk. The mobility of As in soils and water is affected by similar biogeochemical processes as heavy metals and the sources of contamination are also similar (Cheng et al. 2009). For simplification, As and heavy metals will be considered together in this thesis.

Mining activities can especially cause the mobilization of toxic metals, radionuclides, and metalloids from rock materials and soils by dissolution of mineral phases, a drop in pH caused by leaching processes, and the oxidation of sulfidic rock material (Salomons 1995, Dold and Fontboté 2001). These processes can lead to the contamination of pristine areas or

drinking water supplies and consequently, also pose a risk to human health. Other industries, agricultural practices or high natural metal or metalloid abundance, e.g. As, can also cause contaminations, making the contamination of soils, sediments, and water by heavy metals a worldwide problem (Picardal and Cooper 2005, Pedersen et al. 2006).

Metal solubility, mobility and bioavailability in soils is dependent on several parameters, including pH, Eh, speciation, organic carbon content, or the sorption capacity of the soil minerals (Cambier and Charlatchka 1999, Tye et al. 2004, Pareuil et al. 2008). Natural attenuation can immobilize metals and stop their downstream transport by a variety of mechanisms such as sorption, co-precipitation, ion substitution, or redox state transformation (Basta et al. 2005, Picardal and Cooper 2005). Manganese and iron oxides are important scavengers for heavy metals in soils (Mc Kenzie 1989, Schwertmann and Taylor 1989, Basta et al. 2005, Fischer et al. 2007). Although manganese oxides are much less abundant than iron oxides, they often have a higher metal sorption capacity due to their low pH point of zero charge and complex mineralogical structure (Dong et al. 2007). The importance of iron and manganese oxides as sorbents for Co, Ni, Zn, Cu, Cd and Pb has been described for a number of different soils and surface coatings (Latrille et al. 2001, Palumbo et al. 2001, Lee et al. 2002, Liu et al. 2002, Cornu et al. 2005, Dong et al. 2007, Covelo et al. 2007).

Changing geochemical conditions within a soil and the formation of reactive secondary minerals can lead to a substantial natural accumulation of metals, preventing further transport. Such local epigenetic zones, where conditions of element migration are distinctly altered, are called geochemical barriers (Perel´man 1967, Huang and Gong 2005). Geochemical barriers were first described in 1937 by Goldschmidt, who observed an accumulation of selected elements in a coniferous forest which could be ascribed to the presence of humics (Huang and Gong 2005). Geochemical barriers can be formed by chemical, physical, biological, or mechanical means (Perel'man 1967, Leńczowska-Baranek 1996, Huang and Gong 2005) and can also be artificially engineered (Chilakapati 1999).

Microbial influence on heavy metal dynamics in soils

Geochemical processes of metal solubilization and retention can be enhanced or mediated by microbial activity (Benner et al. 1999, Picardal and Cooper 2005). In general, the activity of microorganisms can lead to pH changes in the surrounding environment, thereby altering metal solubility. The reduction of Fe(III)-minerals consumes protons and elevates the pH over time, leading to a decreased solubility of heavy metals. In reducing environments, FeRB and

sulfate-reducing microorganisms can immobilize some metals like Cr and U by direct enzymatic reduction to an immobile and less toxic form (Lovley 1995, Ganesh et al. 1997, Gao and Francis 2008). Metals might also precipitate as metal sulfides with biogenic sulfide produced by sulfate-reducing microorganisms (Machemer and Wildeman 1992, Labrenz et al. 2000). Indirect microbial metal-reduction may also occur via microbially produced reducing agents like Fe(II) and sulfide (Fude et al. 1994, Liger et al. 1999). Reductive immobilization of U, which can be carried out by a phylogenetically diverse assemblage of respiratory and fermentative microbial groups, has been extensively investigated (reviewed in Wall and Krumholz 2006, Wilkins et al. 2006) and used in experimental bioremediation approaches (Anderson et al. 2003, Istok et al. 2004, North et al. 2004). Members of the Fe(III)-reducing *Geobacteraceae* family (δ-*Proteobacteria*) in particular have been demonstrated to be of great importance during uranium-reduction and immobilisation (Holmes et al. 2002, Anderson et al. 2003, North et al. 2004).

On the other hand, metal-reducing microorganisms can also cause metal mobilization by reductive dissolution of mineral phases like iron- and manganese oxides which causes the release of sorbed metals (Francis and Dodge 1990, Cambier and Charlatchka 1999). As(V) may be reduced by FeRB to As(III), increasing its mobility and toxicity (Islam et al. 2005, Tufano et al. 2008). However, sorption and precipitation processes with secondary Fe(II)- and Fe(II)/Fe(III) mixed-minerals can reverse the process, leading to metal retention (Zachara et al. 2002, Cooper et al. 2006). FeRB may also promote the transformation of poorly crystalline Fe(III)-minerals to more crystalline phases, like hematite, goethite, or lepidocrocite (Zachara et al. 2002). Metal sequestration into more crystalline minerals can be enhanced by redox cycling between iron-reducing and iron-oxidizing conditions (Cooper et al. 2006). In oxidizing environments, organisms like Fe(II) and Mn(II) oxidizers can contribute to metal retention by the formation of Fe(III)- and Mn(IV)-oxides which provide new metal sorption sites (Lack et al. 2002, Miyata et al. 2007).

Heavy metal toxicity and metal tolerance

At high concentrations, heavy metals are toxic for microorganisms (Bruins et al. 2000). Toxicity might occur through the displacement of essential metals from functional sites of enzymes, changes in the conformation of proteins and nucleic acids, interference with oxidative phosphorylation and osmotic balance, and the production of oxygen radicals through the Fenton reaction (Bruins et al. 2000, Valko et al. 2005). Microorganisms have

developed resistance mechanisms to deal with heavy metal stress, like metal exlusion by a permeability barrier, efflux systems, intra- and extracellular sequestration, enzymatic detoxification to a less toxic form, and reduced sensitivity of cellular targets (Bruins et al. 2000). Some microorganisms, like *Cupriavidus metallidurans*, *Desulfotomaculum* sp. DF-1, *Escherichia coli*, and *Pseudomonas fluorescens* are highly metal resistant, tolerating mmolar concentrations of selected metals (Fortin et al. 1994, Bruins et al. 2000, Nies 2003, Workentine et al. 2008).

By reductive dissolution of Fe(III)-oxides, FeRB might induce the release of heavy metals from sorption sites. Therefore, Fe(III)-reducers enhance metal availability in their surrounding environment and may be exposed to high metal stress. This suggests that metal tolerance should be an important attribute for FeRB. FeRB are being discussed for use in bioremediation approaches, such as the cleanup of radionuclide contaminated sites (Wilkins et al. 2006), and a high metal tolerance might be necessary to grow in such contaminated soils. However, very little is known about the metal tolerance of FeRB. For example, studies with *Shewanella oneidensis* demonstrated tolerances to only µM concentrations of Cd, Co, Cu, and Zn (Francis and Dodge 1988, Toes et al. 2008). The acidophilic FeRB *Acidiphilium cryptum*, on the other hand, tolerates mM concentrations of Cd, Cu, Ni, and Zn (Dopson et al. 2003). Metal solubility is much higher at acidic than at neutral pH, suggesting that a higher metal tolerance of acidophilic microorganisms might be expected and those organisms might not be comparable with neutrophilic organisms. For the genus *Geobacter*, no metal tolerances are known, although it has been shown that during growth in sediments originating from uranium contaminated sites some proteins are expressed which might be connected to metal resistance (Holmes et al. 2009).

Project Background and Aims

This thesis is part of the Graduate Research School 1257 "Alteration and element mobility at the microbe mineral interface" supported by the German Research Foundation (DFG). The extensive influence of microorganisms on geological and mineralogical processes has been recognized only in the last few decades and is far from being completely understood. The goal of this research group is to investigate the influence of microorganisms on the mechanisms of weathering, reactive transport, and remineralization. In the long run, the experimental data should help to refine strategies for remediation of large catchment areas heterogeneously contaminated with heavy metals.

In the former Ronneburg mining district (Thuringia, Germany), the most productive mining district in the former German Democratic Republic, uranium mining caused severe environmental contamination with heavy metals and radionuclides (Jakubick et al. 1997). In this area two sampling sites were chosen for this thesis (figure 1I).

An acid mine drainage influenced site, "Gessenwiese", has been chosen as a general setting for most of the projects involved in the Graduate Research School. This site is located north of the community of Kauern near Ronneburg on the basement area of the former leaching heap Gessenhalde (figure 1I). Here, low-grade black shale uranium ore, also containing various amounts of Pb-, Zn-, Cu-sulfides, and Ni-arsenides, was leached with acid mine drainage and diluted sulfuric acid between 1971 and 1989 (Wismut 1994, Jakubick et al. 1997). From 1990 on the area was remediated by the WISMUT GmbH, including the removal of the heap material and part of the underlying glacial sediments. However, elevated concentrations of heavy metals remain in the glacial sediments underneath due to infiltration of the leachate through the lining of the heap during operation of the heap. The soil material is a sandy loam and underneath, 1-2.5m below the soil surface, glacial clay occurs. A perched groundwater table is present on top of the glacial clay. The formation of geochemical barriers, like a hardpan, was observed in the vadose zone at about 50 cm depth (Carlsson and Büchel 2005, Grawunder et al. 2009).

The main drainage system of the former mining sites, especially for the leaching heap Gessenhalde and the waste rock dump Nordhalde, is the creek Gessenbach, which is located downstream and was chosen as second sampling site (figure 1II). Consequently, the creek Gessenbach received metal-containing seepage water and surface runoff and might be affected by metals released from the Gessenwiese. During remediation, ascending

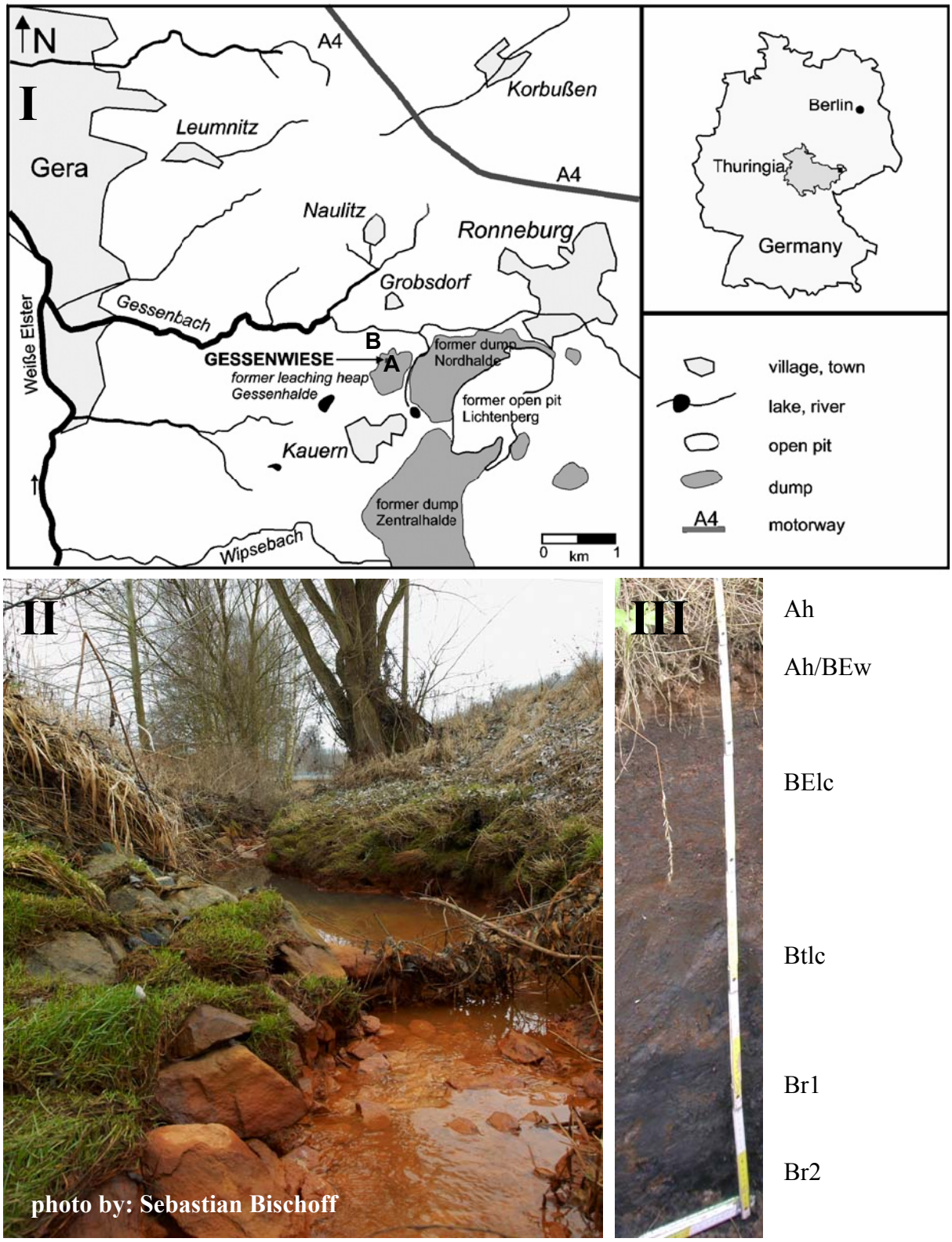

Figure 1: Location of the sampling sites Gessenwiese (A) and Gessenbach (B) within the former mining district Ronneburg in eastern Thuringia, Germany (from Grawunder et al. 2009) (I); the sampling site Gessenbach (II), and the soil profile investigated at the Gessenbach bank soils (III). The names of the soil horizons are given on the right side.

contaminated groundwater from underground mines also leaked out at the Gessenbach (Wismut 2006). Contaminant inflow has strongly declined over the last years as a result of the remediation of the mining sites (Wismut 2006) but contaminants enriched over the last decades remain in the gleyic soil (Schmidt et al. 2009b). At the banks of the creek Gessenbach, horizons with black material are present, which may act as geochemical barriers (figure 1III). The permanent exposure to high metal concentrations during the last 50 years might have promoted the development of organisms with increased metal tolerance (Bruins et al. 2000, Kothe et al. 2005), that may contribute to metal retention in geochemical barriers.

In my thesis, I investigated the impact of FeRB on heavy metal retention and the effect of heavy metals on the process of Fe(III)-reduction in contaminated soils and glacial sediments at both Ronneburg sampling sites, Gessenbach and Gessenwiese and tested the following hypotheses:

I. FeRB support the formation of metal-retaining iron- and manganese-rich geochemical barriers in groundwater-affected soils and glacial sediments.

II. Indigenous FeRB are tolerant to heavy metal stress.

The objectives of this thesis were:

I. To characterize soil profiles at the sampling sites Gessenwiese and Gessenbach, which contain geochemical barriers, biogeochemically by pore water analysis, total and sequential extraction of the soil solid phase, total cell counting and most probable number (MPN) analysis,
II. To identify the dominant anaerobic microbial redox processes in the geochemical barriers,
III. To link Fe(III) reduction with heavy metal dynamics and to identify the associated microbial community via SIP, terminal restriction length polymorphism (TRFLP), cloning, and sequencing,
IV. To evaluate the heavy metal tolerance of the indigenous FeRB community, and
V. To identify a model Fe(III)-reducing bacterium, JF5, via means of Raman spectroscopy and to use this technique to differentiate between (i) different growth phases, (ii) aerobic and anaerobic metabolism, (iii) growth in presence of heavy metals.

Thesis Structure

In the **first chapter** *"Effect of metal-reducing microorganisms on element fluxes in a former uranium-mining district"* the geochemistry and the microbial activities at both sampling sites, Gessenwiese and Gessenbach are reviewed. A detailed characterization of the geochemical barriers at the Gessenwiese is presented in the **second chapter** "*Heavy metal retention and microbial activities in geochemical barriers formed in glacial sediments subjacent to a former uranium mining leaching heap"*, including total and sequential extraction data, pore-water chemistry, microbial abundances, and microbial activities in anoxic soil microcosms.

The next three chapters are dealing with the sampling site Gessenbach. The impact of microbial Fe(III)-reduction on metal retention and the associated microbial communities are discussed in the **third chapter** *"Impact of biostimulated redox processes on metal dynamics in an iron-rich creek soil of a former uranium mining area"*, while the **fourth chapter** *"Microbial links between sulfate reduction and metal retention in uranium- and heavy metal-contaminated soil"* links the activity of sulfate-reducing microorganisms with metal-dynamics. Both manuscripts present results from SIP-experiments and include data of the pore-water chemistry and the speciation of uranium. The **fifth chapter** *"Metal tolerance of Fe(III)-reducing microbial communities in a contaminated creek soil*" describes the sensitivity of microbial Fe(III)-reduction to heavy metal contamination and provides information about the Fe(III)-reducing microbial communities in the presence of heavy metals. In addition to the results presented in this manuscript, data on the acidophilic FeRB *Acidiphilium cryptum* JF-5 are included in the General Discussion section.

The **sixth chapter** *"The influence of intracellular storage material on bacterial identification by means of Raman spectroscopy"* evaluates the potential of Raman spectroscopy as a method for the identification of FeRB. This work was done in cooperation with Valerian Ciobotă (Institute of Physical Chemistry). In particular, the influence of the storage compound PHB on microbial identification is discussed. Additional data on the differentiation of metabolic states of the cells by means of Raman spectroscopy are provided in the General Discussion section.

Effect of metal-reducing microorganisms on element fluxes in a former uranium-mining district

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Abstract

Metal reducing microorganisms influence the speciation of metals in soils by different direct and indirect mechanisms. Metal-retention occurs in geochemical barriers in two sites of the former uranium mining district Ronneburg (Thuringia, Germany). This manuscript reviews the geochemistry of these geochemical barriers and the microbial impact on their formation and stability.

Metal-reducing microorganisms and metal mobility. A wide range of microorganisms are capable to conserve energy for growth via the reduction of Fe(III) to Fe(II) (Lovley et al., 2004). Many of these microorganisms can also grow via the reduction of Mn(IV) to Mn(II). The first organisms shown to grow with Fe(III) or Mn(IV) as the sole electron acceptor were *Shewanella oneidensis*, *Shewanella putrefaciens*, and *Geobacter metallireducens* (Myers and Nealson 1988, Lovley et al., 1987). Geochemical and microbial evidence suggest that reduction of Fe(III) is an early form of respiration of Earth (Vargas et al., 1998). The ability to oxidize hydrogen with the reduction of Fe(III) is highly conserved among deeply branching hyperthermophilic *Bacteria* as well as *Archaea* that are most closely related to the last common ancestor(s). Today, more than 90 species have been characterized (Lovley et al., 2004) including psychrotolerant and hyperthermophilic, neutrophilic and acidophilic microorganisms (Küsel et al., 1999; Kashefi et al., 2003). At circumneutral pH, Fe(III) exists predominantly as oxyhydroxide mineral and is highly insoluble in contrast to low pH conditions (Schwertmann and Taylor, 1989). The energetics of Fe(III) reduction under pH neutral conditions differ substantially from those under acidic conditions. Thus, it is not surprising that Fe(III) reduction in pH neutral and acidic environments is carried out by different microbial populations (Straub et al., 2001). Many fermentative microorganisms also reduce Fe(III) as a minor side reaction in their metabolism, but they do not appear to gain energy from this electron transfer. Some dissimilatory sulfate-reducing and methanogenic microorganisms can reduce Fe(III), but growth has not been unambiguously demonstrated using these electron acceptor (Lovley et al., 2004). In many recent subsurface environments, Fe(III) respiration is coupled to a substantial portion of organic matter mineralization (Lovley and Philipps, 1988), and Fe(III) reduction is an important process catalyzing a large number of natural and contaminant biogeochemical cycles.

Fe(III)- and Mn(IV)-reducing microorganisms can influence the mineralogy of sediments through the reductive dissolution of insoluble Fe(III) and Mn(IV) oxides, which leads to the release of potentially toxic levels of Fe(II) and Mn(II), and also of trace metals that were adsorbed to the former minerals (Lloyd 2003). Metal- reducing microorganisms can also affect multivalent metals and radionuclides that pose environmental problems (e.g. Lovley et al., 1991; Fude et al., 1994; Lloyd et al., 2000, Myers et al., 2000; Istok et al., 2004) through direct enzymatic reduction, or via indirect reduction catalsed by biogenic Fe(II) or H_2S (Fig. 1). The toxic oxidized forms of uranium, technetium, and chromium are highly soluble in aqueous media and mobile in oxidized aquifers, while the reduced species are

highly insoluble and often precipitate from solution. In its most oxidized state, uranium [U(VI)] forms very stable aqueous complexes (e.g. carbonate, hydroxide), which greatly increase its solubility and mobility in aqueous habitats. Several dissimilarory Fe(III)-reducing microorganisms have been reported to reduce hexavalent uranium [U(VI)] to tetravalent uranium [U(IV)] and precipitate a U(IV) mineral called uraninite (UO_2). At least two have been shown can grow with U(VI) as sole electron acceptor (Lovley et al., 2004). Also sulfate-reducing bacteria, like *Desulfovibrio desulfuricans*, *Desulfovibrio vulgaris*, and *Desulfotomaculum reducens* sp. nov. strain MI-1, and fermentative bacteria have the capacity to reduce U(VI). Reductive precipitation of uranium via microbial U(VI) reduction is effective in removing uranium both in contaminated laboratory reactors and in the *in situ* treatment of contaminated groundwaters (Anderson et al., 2003; Istok et al., 2004). In contrast to these metals, arsenate (As(V)) binds strongly to sediments under oxic conditions. The reduction of As(V) leads to the more mobile and toxic As(III), which contaminates wells of drinking water in the Ganges-Meghna-Brahmaputra delta plain (Lloyd 2003).

Microorganisms can also enhance metal retention indirectly by formation of reducing agents like Fe(II) or sulfide. Sulfide can react with metals to form insoluble products. Sphalerite (ZnS) particles formed within biofilms dominated by sulfate reducers can concentrate other metals like arsenic and selenium (Labrenz et al., 2000). Thus, microbial precipitation reactions can be central to mine-waste remediation strategies using natural or artificial wetlands (Machemer and Wildeman, 1992). Research is driven forward both by the need to understand biogeochemical cycles of several key elements, and also by the possibility of harnessing microbial activities for a range of biotechnological applications, such as bioremediation of metal-contaminated soil and aquifers. Our studies aim to achieve a mechanistic understanding of the coupling between microbial iron metabolism, chemical and physical reactions at mineral surfaces, and reactive metal transport in the former Uranium-mining district Ronneburg.

Uranium-mining district Ronneburg. In the former uranium mining district Ronneburg (eastern Thuringia, Germany) mining activities caused severe environmental contaminations with heavy metals, radionuclides and acid mine drainage. Uranium mining in the former German Democratic Republic was extensive during the period 1946–1990 with a total production of over 200 kt of uranium (Jakubick et al., 1997). More than half of the uranium production originated from the Ronneburg operations and heap leaching at the Gessenhalde

site, where low-grade black shale uranium ores were leached (Carlsson and Büchel, 2005). Before placement of the low-grade ore a foundation layer and a barrier soil consisting of compacted loam were constructed, and then acid mine water (pH 2.7-2.8) and diluted sulfuric acid were used between 1971 and 1987 to enhance the shale leaching process. In 1989, remediation activities in Ronneburg started, but remediation actions, such as groundwater treatment, will continue for several decades (WISMUT 1994a,b; WISMUT 1997).

Ore samples from 1993 within the Gessenhalde leaching heap prior to remediation still showed acidic conditions (pH 4) and high concentrations of Fe, Mn, Cu, and Zn approximating 250, 75, 20, and 18 mg/kg, respectively (Schippers et al., 1995). Thus, the leaching material was relocated to an open pit and mixed with anhydrous lime. Also soil underneath the formed dump was removed and the remaining sandy loam overlaying glacial clay was seeded using a grassland seed preparation (Carlsson and Büchel, 2005). On top of the glacial clay a perched groundwater table exists. Secondary Fe precipitates, mainly goethite, were observed in the loam forming patchy dense, cemented layers which are designated as hardpan. The goethite is enriched with elements like Cd, Ni, As, and U apparently originating from the minerals present in the black shales (Geletneky et al., 2002). Thus, leachate appeared to infiltrate since the 70ties through the barrier soil into the loam and was trapped on top of the glacial clay. These hardpans act as geochemical barriers, because selected elements are retained in these epigenetic zones and the further element transport is inhibited (Huang and Gong, 2005). Thus, we have to asses the stability of these geochemical barriers for the future mobility of the heavy metals adsorbed or co-precipitated to the secondary Fe minerals.

During the leaching period, the pH should have been between 2.8 and 4 leading to the formation of Fe-hydroxosulfates, presumably schwertmannite [$Fe_8O_8(OH)_x(SO_4)_y$] as the dominating secondary Fe precipitate of this pH range (Bigham et al., 1996a). Schwertmannite is known to age into the more crystalline mineral goethite (Bigham et al., 1996b), a process occurring also in sediments of acidic coal mining lakes (Peine et al., 2000). Upon ageing into goethite, sulfate is released accompanied with a decrease in pH which leads to a mobilization of previously adsorbed metals. However, metals can also remain in the mineral phase during the transformation to geothite (Schroth and Parnell Jr., 2005). Oxidized uranium, present as UO_2^{2+}, might not have been scavenged extensively by Fe precipitates due to its high mobility. After the complete transformation of schwertmannite, the acid-producing capacity will be lost, and the remaining solid fractions will be less mobile in the groundwater under oxic

conditions (Carlsson and Büchel, 2005). The revegetation of this area will provide an increase of soil organic matter resulting in enhanced respiratory activity, which might enable the reductive dissolution of goethite by Fe(III)-reducing microorganisms under anoxic conditions. During the reductive dissolution, not only Fe(II) but also adsorbed metals might be mobilized. However, the microbial reduction of Fe minerals consumes protons. The pH increase might inhibit further element mobility and improve the adsorption of metals onto other mineral surfaces like clay minerals, especially illite. Thus, the importance of clay minerals in adsorbing metal cations are likely to increase in the future with higher primary biomass production.

Microbial activities in the former Gessenhalde leaching heap. In 2006, microbial respiratory activities in the loamy soil were still low. Total cell numbers of microorganisms were below 10^6 cells g $(soil)^{-1}$. The pH of the soil water approximated 4. Concentrations of nitrate in the soil solution did not decrease with increasing soil depth and averaged 100-to-200 µM. Anaerobic activities could only be initiated after the addition of organic carbon in soil microcosms. Surprisingly, depth profiles of soluble heavy metals peaked above the Fe-precipitate layer in a cemented greyish horizon. This layer was dominated by manganese oxides as confirmed by X-ray powder diffraction analysis. Sequential extraction analyses demonstrated that especially Mn-oxides were important sorbents for heavy metals like Cd, Ni, Zn, and Cu. Thus, two geochemical barriers were identified that retained metals either in Mn-oxides or in Goethite indicating stable retention mechanisms. Since the deeper soil is still carbon-limited, oxic conditions will remain in the near future in the loam, but inflowing shallow groundwater from the catchment area will disperse and wash out the mobile fractions of the heavy metals.

Geochemical barriers at the Gessen creek. Groundwater from the former Gessenhalde leaching heap enters a small creek, named Gessen creek. The Gessen creek runs from the village Kauern between the two former waste rock dumps Nordhalde and Gessenhalde first into the Weisse Elster and then into the Elbe and North Sea. The water chemistry is dominated by $Fe/SO_4/Mg$, but the creek water loses much of its Fe content due to Fe(III) hydroxide formation and precipitation (Kothe et al., 2005). Today, the pH of the creek water approximates 6-to-7, but concentrations of sulfate still reach 2-to-8 mM. The creek infiltrates the surrounding soil. The banks of this creek appear to be enriched with *Streptomyces* strains

cabable to withstand high concentrations of heavy metals (especially Ni) indicating an adaptation during the last 40 years of contamination (Schmidt et al., 2005; Kothe et al., 2005). Similarily, uranium mining waste piles heavily polluted with toxic metals are shown to be reservoirs for bacteria that have evolved special strategies to survive in these extreme environments; e.g. cells of *Bacillus spaericus* JG-A12, isolated from a waste pile, accumulate high amounts of toxic metals such as U and Cu, which are bound by a highly ordered paracrystalline proteinaceous surface layer that envelopes the cell (Pollmann et al., 2006). These special capabilities of the cells are interesting for clean-up of uranium contaminated waste waters.

The gleyic soils at the bank of the Gessen creek influenced by the groundwater flow from the former Gessenhalde leaching heap are slightly acidic (pH 5-6) and have a sandy silty texture (Table 1). In general, the subsurface respiratory activities were 100 times higher than at the Gessenhalde leaching heap. The organic carbon content can reach up to 6 % in the oxidized groundwater influenced horizon (GoII: 80-100 cm soil depth), but the basal respiratory activities were not correlated with the organic carbon contents. In the reduced groundwater influenced horizon (GrII: 130-140 cm soil depth), the redox potential is low (-30 mV), and total amounts of sulfur reach 2%. Total numbers of microorganisms were highest in the reduced GrII horizon with 10^9 cells g $(soil)^{-1}$. The solid phase of the GoII and GrII horizons were highly enriched with Fe, Mn, U, Zn, Ni, Pb, and Cd suggesting that both horizons act as geochemical barriers (Zelenova et al., 1970). The GoII horizon was Al, Zn, and Ni peaked in the soil solution of the GoII horizon, and Mn, and U in the soil solution GrII horizon. Sequential extractions showed that 6-to-18% of all elements were bioavailable; especially Cd, Ni, U, and Zn occurred in the mobile fraction. The organic bound fraction contained 16 and 12% of the total amount of heavy metals in the GoII and GrII horizon, respectively. Mn-oxides appeared not to be important for metal retention. Up to 40% of the metals were bound to Fe-oxides; especially less crystalline Fe-oxides scavenged three times more elements than the better crystalline fraction. Thus, reductive dissolution of these Fe-oxides should affect the metal mobility in these soils.

Microbial reduction of Fe(III) in contaminated gleyic soils. According to their high heavy metal retention capacity, the GoII and GrII horizon were investigated in more detail. When soil of the GoII horizon was incubated under anoxic conditions, Fe(II) formation was observed only in those microcosms amended with carbon sources like ethanol, lactate, and

glucose. When heavy metals were added in concentrations which matched those of the mobile and bioavailable fractions, Fe(II) formation could not be stimulated even in the presence of supplemental carbon sources. Arsenic but also uranium were mobilized during Fe(III) reduction, whereas Ni and Zn concentrations decreased. Fe(III) reduction followed nitrate consumption which is present in the GoII horizon only in small concentrations (approximately 50 µM). The low concentrations of nitrate are in contrast to other uranium-contaminated aquifers in the U.S. nuclear weapons complex managed by the Department of Energy (DOE). In the DOE´s Environmental Remediation Sciences Program Field Research Center (ORFRC), in Oak Ridge, Tennessee, the combination of low pH and high concentrations of radionuclides and nitrate (up to 500 mM) is representative. Members of the *Deltaproteobacteria* (*Geobacter* spp., *Anaeromyxobacter* spp.) appear to be important metal-reducers in acidic subsurface sediments upon pH neutralization and carbon substrate addition (North et al., 2004). Little or no U(VI) reduction seems to occur in co-contaminated sediments in the presence of nitrate, and only after nitrate depletion, U(VI) and Fe(III) are reduced concurrently (Finneran et al., 2002a, b). Due to the stress imposed by low pH on microbial metabolism, terminal electron accepting processes are inherently different from those of neutral pH environments and neutralization appears to be necessary in the acidic subsurface sediments collected from ORFRC to achieve sufficient metabolic rates of radionuclide remediation (Edwards et al., 2007).

Aquifers at the former uranium-mining district Ronneburg are only slightly acidic but have to face elevated heavy metal concentrations derived from the leaching of the low-grade black shale. Most probable numbers of Fe(III) reducers cultured at pH 5.5 with goethite as electron acceptor were 3 orders of magnitude lower in the presence of heavy metals correspondent to *in situ* concentrations. PCR with DNA extracted from different horizons yielded products with 16S rRNA primers specific for acidophilic Fe(III) reducers belonging to *Acidiphilium* only in both slightly acidic Go horizons. Primer sets specific for *Geobacter* yielded products in all horizons, whereas products specific for *Shewanella* were detected only in upper horizons. No PCR products were obtained with a primer set specific for *Geothrix*. The diversity of the Fe(III)-reducing community matches with those obtained from other acidic environments, like sediments of coal mining lakes (Blöthe et al., 2007) or even acidic peatlands, suggesting that the pH is a crucial parameter thriving microbial processes.

In contrast to GoII, Fe(II) was formed with high rates in unsupplemented soil microcosms of the GrII. The spontaneous Fe(II) formation and the high initial Fe(II)

concentrations indicated that Fe(III) reduction is an on-going process in the GrII horizon. Surprisingly, none of the carbon sources tested (ethanol, lactate, acetate, glucose) could increase the rate of Fe(II) formation. The added carbon sources were utilized concomitantly to sulfate reduction which followed the reduction of Fe(III) (Fig. 2). The reduction of sulfate was mostly coupled to acetate oxidation. Again, As and U were mobilized during the reductive processes, and Ni and Co concentrations decreased. Pyrite was the dominating Fe sulfidic mineral.

Our data suggest that the banks of the Gessen creek act as geochemical barriers for many heavy metals. Especially the Fe-oxide fraction in the GoII horizon appears to be a stable geochemical barrier. Nonetheless, parts of As and U can be mobilized and will enter the Gessen creek. Oxic condition might prevail in the GoII horizon *in situ* due to the relatively low aerobic soil respiration activity despite the high amounts of organic carbon present. The high concentrations of bioavailable heavy metals present in the GoII horizon appear to inhibit also the microbial reduction of Fe(III), and reductive dissolution of Fe-oxides accompanied by a mobilization of U, Pb, and Ni that are bound to the less crystalline Fe-oxides is not be expected. Reductive processes, especially Fe(III) and sulfate reduction, appear to occur in the GrII horizon. The activity of the sulfate reducers which receive sulfate both from the groundwater flow and from the inflitrating creek water might help to overcome the metal toxicity by co-precipitation of the heavy metals. Additionally, the resident sulfate-reducing community might dispose of molecular resistance mechanisms which have to be elucidated. The mobilization of uranium in the carbon amended soil microcosms is not understood, because uranium released during reductive mineral dissolution should be subjected to microbial reduction and precipitate as uraninite. Uranium concentrations peaked also in the soil solution of the GrII indicating the *in situ* relevance of uranium solubility in reduced horizons. Thus, further research is needed to understand the uranium chemistry in contaminated habitats.

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Table 1: Geochemical characteristics of the subsurface soil at the bank of the Gessen creek.

Horizon	Depth [cm]	Texture	pH	Eh [V]	C [%]	N [%]	S[%]	Fe [mg/g]
Al	22 -41	silt	n.d.[a]	n.d.	0.32	0.01	0.03	23.3
GoI	41 – 80	sandy silt	5.9	n.d.	4.97	0.34	0.18	69.7
GoII	80 -101	clayey silt	4.9	0.27	5.95	0.36	0.37	65.3
GrI	101 – 129	sandy silt	5.2	0.06	3.42	0.17	0.42	48.0
GrII	129 - 137	sandy silt	6.1	-0.03	3.43	0.18	1.98	66.1

[a] n.d.: not determined

Figure Legends:

Fig. 1: Model of microbial direct (A) and indirect (B) reduction of heavy metals. Electrons are derived from oxidation of an electron donor, e.g. acetate, to CO_2.

Fig. 2: Formation of Fe(II) and consumption of sulfate and acetate in anoxic GrII soil microcosms. Concentrations of dissolved heavy metals at the beginning and at the end of incubation.

Figure 1

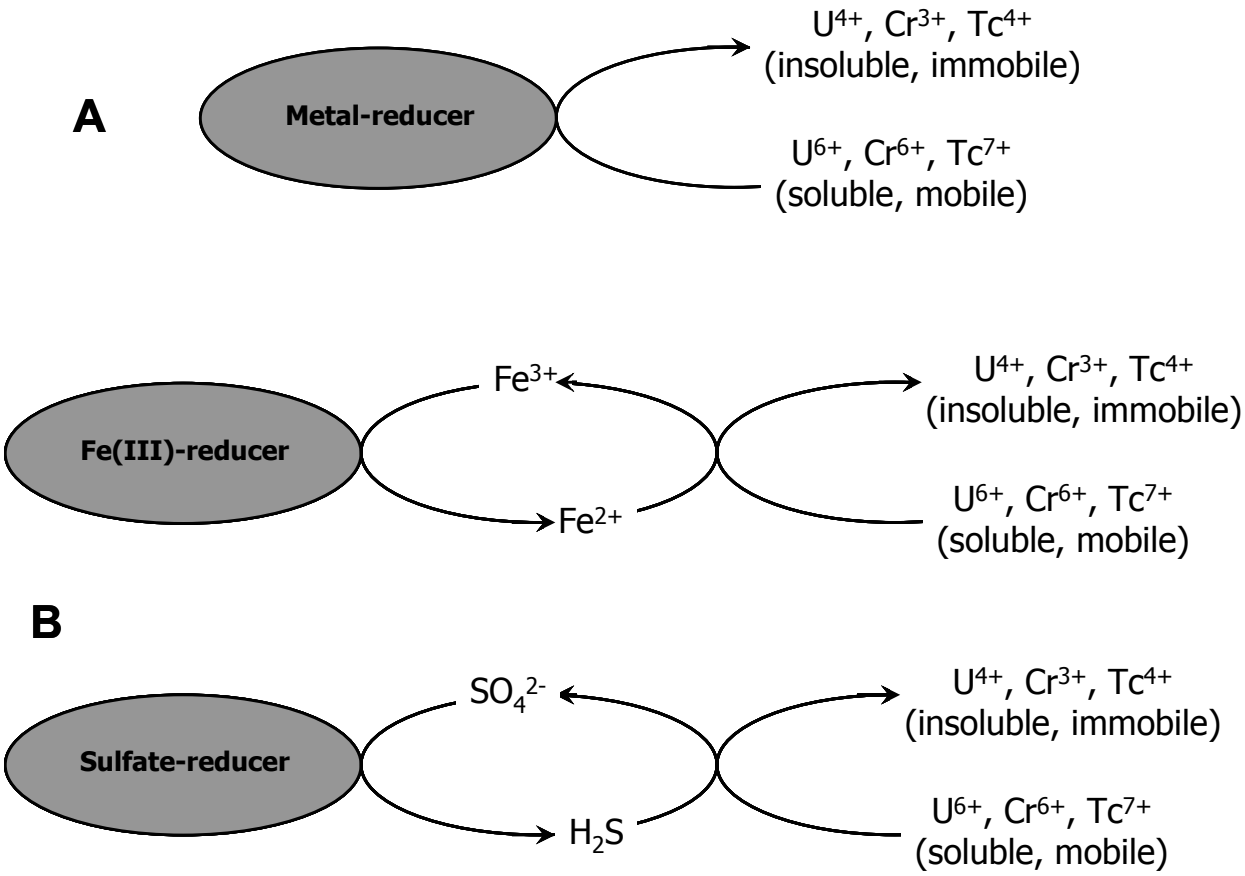
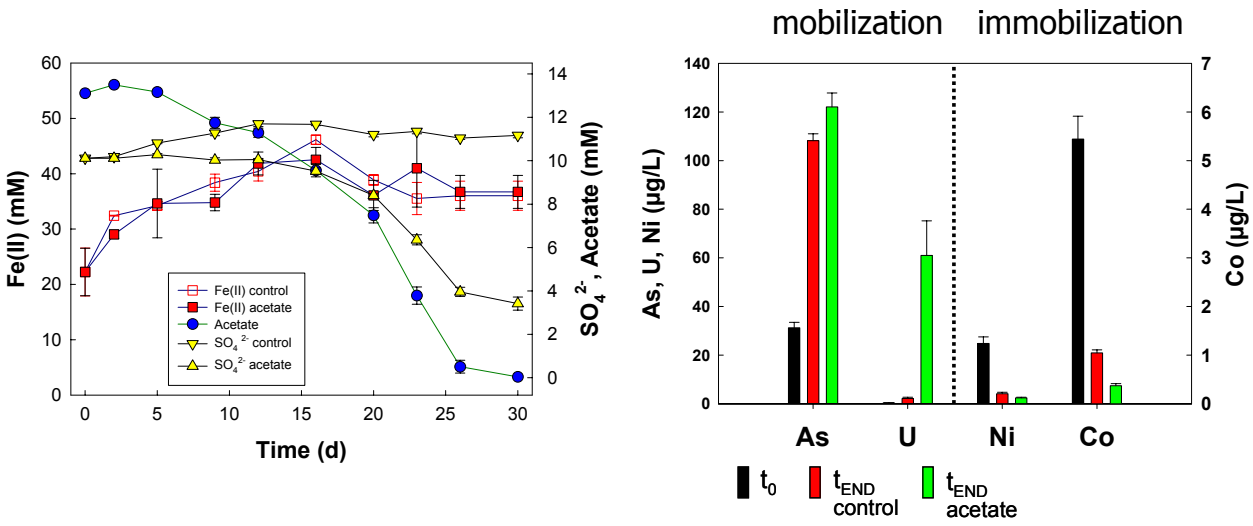

A
Metal-reducer
U4+, Cr3+, Tc4+
(insoluble, immobile)
U6+, Cr6+, Tc7+
(soluble, mobile)
Fe(III)-reducer
Fe3+
Fe2+
U4+, Cr3+, Tc4+
(insoluble, immobile)
U6+, Cr6+, Tc7+
(soluble, mobile)
B
Sulfate-reducer
SO4 2-
H2S
U4+, Cr3+, Tc4+
(insoluble, immobile)
U6+, Cr6+, Tc7+
(soluble, mobile)

Figure 2


Fe(II) (mM)
SO_4^{2-}, Acetate (mM)
Time (d)
Fe(II) control
Fe(II) acetate
Acetate
SO_4^{2-} control
SO_4^{2-} acetate
mobilization
immobilization
As, U, Ni (µg/L)
Co (µg/L)
As
U
Ni
Co
t_0
t_{END} control
t_{END} acetate

Heavy metal retention and microbial activities in geochemical barriers formed in glacial sediments subjacent to a former uranium mining leaching heap

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Abstract

Understanding the co-precipitation and sorption of metals and radionuclides in minerals and the biologically induced release of metals is important for predicting the future contaminant mobility in sediments affected by groundwater flow. This study aims to determine the retention of heavy metals in local epigenetic zones, termed geochemical barriers, in sediments of a former uranium mining district (Ronneburg, Germany) and to evaluate the potential microbial impact on the formation and stability of these barriers. Accumulation of heavy metals occurred in two distinct geochemical barriers. Cd, Ni, and Co accumulated in a greyish, manganese rich layer, whereas the metalloid As, and the metals U, Ni, and Co accumulated in a reddish-brown, iron rich layer. Crystalline iron oxides, mainly goethite, served as important heavy metal sorbents in the reddish-brown, iron rich layer. Iron and manganese oxides occurred mainly as cover on quartz grains and as cement between the quartz grains which might further close the porous sandy sediment matrix. On a molar basis, the manganese oxide barrier appeared to be more effective in metal retention than the iron oxide barrier. The presence of nitrate and Fe(III) in the porewater indicated oxic conditions throughout the sediment profile. Microbial abundances and activities in the sediment profile were low, apparently due to the low organic carbon content of the sediment but not due to toxic effects of heavy metals. Nitrate- and Fe(III)-reducing activities could be detected only after the addition of a carbon source. Collectively, these results demonstrated that both barriers prevent contaminant mobility in the aquifer and that reductive microbial processes did not have a major impact on metal retention at this site.

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Heavy metal retention and microbial activities in geochemical barriers formed in glacial sediments subjacent to a former uranium mining leaching heap

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Abstract

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Keywords: Heavy metals; Uranium; Metal retention; Sequential extraction; Geochemical barrier; Mining; Microbial iron reduction; Manganese oxide; Goethite; Heavy metal tolerance

1. Introduction

Environmental contamination with heavy metals and metalloids due to mining activities is of great concern as heavy metals can enter the food chain and are toxic to organisms including humans (Giri et al., 1980; Sharma and Agrawal, 2005). The leaching of ore material with

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acids and the aerobic oxidation of sulfidic rock material cause a pH decrease that increases heavy metal mobility and leads to contamination of pristine areas (Salomons, 1995; Dold and Fontboté, 2001).

Heavy metals and metalloids can be retained in soils or sediments by mechanisms like sorption, co-precipitation, ion substitution, and redox state transformation (Machemer and Wildeman, 1992; Basta et al., 2005; Picardal and Cooper, 2005; Huang and Gong, 2005). Manganese and iron oxides are important scavengers for heavy metals in soils (Mc Kenzie, 1989; Schwertmann and Taylor, 1989; Basta et al., 2005). The importance of manganese oxides as sorbents for Cd, Co, Cu, Ni, Pb, and Zn is described for different soils and surface coatings (Latrille et al., 2001; Palumbo et al., 2001; Liu et al., 2002; Cornu et al., 2005; Covelo et al., 2007; Dong et al., 2007). Thus, the formation of reactive secondary minerals can lead to a substantial natural accumulation of selected elements in local epigenetic zones, called geochemical barriers, where conditions of element migration are distinctly altered (Perel'man, 1967; Huang and Gong, 2005). Geochemical processes leading to the formation or dissolution of geochemical barriers can be enhanced or mediated by microbial activities (Benner et al., 1999; Picardal and Cooper, 2005). During the reductive dissolution of manganese and iron oxides by anaerobic microorganisms, adsorbed heavy metals can be released (Francis and Dodge, 1990; Cambier and Charlatchka, 1999; Charlatchka and Cambier, 2000). Metal- and sulfate-reducing microorganisms can also immobilize some metals by direct enzymatic reduction to an immobile reduced form, whereas the microbial reduction of arsenate leads to the formation of a more mobile species (Lovley et al., 1991; Ganesh et al., 1997; Picardal and Cooper, 2005). Microorganisms can also cause metal reduction indirectly through production of metal reductants such as Fe(II) or sulfide (Machemer and Wildeman, 1992; Fude et al., 1994; Liger et al., 1999; Labrenz et al., 2000). Under changing redox conditions such metal accumulations can be remobilized by chemical or microbiological mechanisms (Francis and Dodge, 1990; Cambier and Charlatchka, 1999; Charlatchka and Cambier, 2000).

In the former mining district Ronneburg (Thuringia, Germany), one of the most important mining districts in the former German Democratic Republic, uranium mining caused severe environmental contaminations with heavy metals including radionuclides (Jakubick et al., 1997). One of the production sites was the leaching heap Gessenhalde where low-grade black shale uranium ore was leached with acid mine drainage (pH 2.7 2.8) and diluted sulfuric acid between 1971 and 1989 (Wismut, 1994a, b). The remaining heap material was removed during remediation. However, elevated concentrations of heavy metals remained in the glacial sediments underneath due to infiltration of the leachate through the lining of the heap during the mining activities (Carlsson and Büchel, 2005; Grawunder et al., 2008, this issue). Increased concentrations of As, Cd, Ni, and U occur in a patchy cemented layer (hardpan) of a sandy loam close to a shallow aquifer. The hardpan consists mainly of goethite, which is presumed to be the main metal scavenger at this site (Carlsson and Büchel, 2005). However, manganese oxides often are more important for heavy metal accumulation than iron oxides due to their large surface area and low point of zero charge (Mc Kenzie, 1989; Dong et al., 2007). The formation of geochemical barriers like cemented layers or hardpans are also described in low sulfide and low carbonate Freiberg polymetallic mine tailings (Graupner et al., 2007). Hardpans consist mainly of iron oxides and the highest concentrations of originally dissolved metals are observed above or within the hardpan layer, because their low permeability reduces metal movement in an oxic environment. The porosity is reduced by precipitation of secondary phases and gels, which may coat and agglutinate particles (Graupner et al., 2007). Cemented layers are often observed at the transition between oxidized and reduced zones with strong variations in pH and Eh (Graupner et al., 2007). Thus, precipitation will close the porous space, limit the aeration regime, and might promote the activity of anaerobic metal reducing microorganisms.

While an accompanying paper describes the grade, distribution, and the bonding of residual contaminations in these sediments on a larger spatial scale (Grawunder et al., 2008, this issue), this study aims (i) to determine the heavy metal distribution and natural attenuation in a sediment profile with an iron-rich layer located near the aquifer, (ii) to evaluate the potential importance of iron and manganese oxides acting as scavengers for other metals, and (iii) to elucidate the potential activity of anaerobic microorganisms which might affect metal mobility in the geochemical barriers near the groundwater table. Thus, analyses of the sediment solid phase, sequential extractions, porewater biogeochemical and microbiological analyses were combined to estimate the bioavailability and the future fate of metal contaminants at this site.

2. Materials and methods

2.1. Study site and sampling

The study site is located on the area of the former uranium leaching heap Gessenhalde near Ronneburg (Thuringia, Germany). A more detailed description of the construction of the leaching heap Gessenhalde, the situation after leaching, and the reconstructed profile of the northern area of the

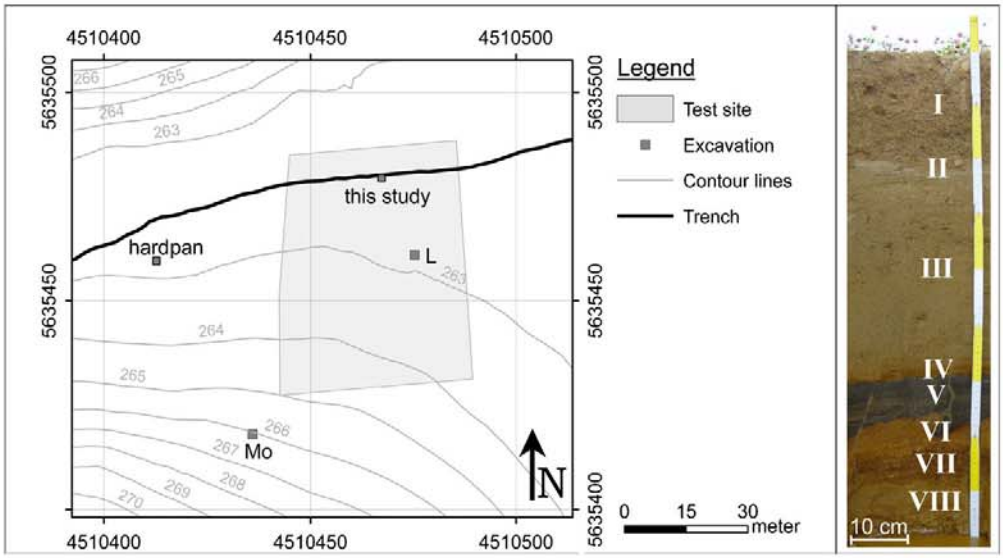

Fig. 1. Map of the test site Gessenwiese (left) and the sediment profile investigated (right). The map presents the sampling locations of the profile investigated in this study, the hardpan sample studied by Carlsson and Büchel (2005), and the excavations "L" and "Mo" analyzed in Grawunder et al. (2008, this issue).

Gessenhalde is presented elsewhere (Grawunder et al., 2008, this issue). Quaternary sediments subjacent to the dumped Ordovician and Silurian black shales were contaminated by penetration of the leachate through the drainage and barrier layers. During remediation, the dump material and up to 10 m of the excavated underground material were removed and the basement area was recontoured using an allochthonous soil as filling material. Since 2004, a part of the Gessenhalde, the test field "Gessenwiese", is used as a platform for applied and basic research activities dealing with phytoextraction, mobilization, and alterations of minerals.

At the test field, several local epigenetic zones were discovered that consist of strata-bound dm-thick black to dark reddish layers consisting of iron and manganese oxides and hydroxides, barium and manganese hydroxide, and clay minerals. The sediment profile studied (Fig. 1) is located 55 m northeast of a formerly described profile (Carlsson and Büchel, 2005). The surface layers I and II (Table 1) were deposited as filling material, and soil formation has started approximately 10 years ago.

Sampling was done in June and July 2006 with exception of samples for most probable number (MPN) analyses which were taken in November freshly for the experiment. Solid-phase sediment samples for chemical analyses and microcosms were collected from each layer and stored in plastic bags in the dark at 4 °C for two weeks prior to the onset of microcosm experiments. Sediment water samples were obtained under water-saturated conditions between 58 and 84 cm depth using a dialysis sampler (Schmalenberger et al., 2007). The sampler was equipped with a cellulose acetate membrane filter with a pore size of 0.2 µm (OE 66, Schleicher and Schuell). Before displacement, the dialysis sampler was filled with anoxic (N_2 gassed) distilled water. After 13 days of equilibration with the porewater, the dialysis sampler was removed, transported to the laboratory, and samples were analyzed immediately. Samples for direct counts of microbial cells or for MPN estimates were each collected from one core of a Pürckhauer-corer using sterile techniques and were processed 3 days or 6 days later, respectively. Groundwater for microcosms and MPN analyses was obtained from the trench in front of the profile to ensure its similarity to the pore water sampled with the dialysis sampler.

2.2. Analysis of sediment samples

Sediment pH was measured using the $CaCl_2$ method as previously described (Grawunder et al., 2008, this issue). Field-fresh duplicate sediment samples were dried at 105 °C for 24 h to determine the water content. Sediment organic matter (C_{org}) was analyzed by loss-on-ignition at 500 °C for 4 h. Sediment samples were air-dried and sieved (<2 mm) and grain size was determined by laser diffractometry with an LS 13320 (Beckman Coulter, Fullerton, CA, USA) following the procedure ISO 13320-1 (1999). Humic substances in the samples were removed by the addition of H_2O_2 (10% (v/v)). For CNS analysis, determination of total element content, and analyses of minerals, sediment samples were air dried, sieved (<2 mm), and finely ground (<63 µm) with a centrifugal ball mill (S100, Retsch, Hahn, Germany) for 45 min. CNS analysis was done in a CHNOS elementary analysator VARIO EL (Elementar Analysensysteme, Hanau, Germany). Sediment minerals were determined using an X-ray powder diffractometer XRD7 (Seifert-FPM, Freiberg, Germany, with rayflex software, Cu Kα radiation, 40 kV and 30 mA) at a scanning span 2θ of 5–70° in steps of 0.02°. Analysis was done with software MacDiff (Apple Macintosh) and the internet-database at http://rruff.geo.arizona.edu/AMS/amcsd.php.

Table 1. Sediment characteristics.

Layer	Depth (cm)	Color	Sediment pH ($CaCl_2$)	Sediment water content (% ww[a])	C_{org} (% dw[b])	Basal respiration rates (nmol CO_2 g(ww)$^{-1}$ h^{-1}) ±standard deviation	Grain size distribution (%wt)							
							G[c]	cS[d]	mS[e]	fS[f]	cU[g]	mU[h]	fU[i]	T[j]
I	0–21	Yellowish-brown 10 YR 5/6	4.3	9.0	1.1	9.2±0.92	0.3	1.9	48.0	25.0	0.2	14.4	8.1	2.2
II	21–24	Grey-brown 10 YR 6/4	4.0	26.5	0.8	4.6±0.81	<0.1	<0.1	17.5	58.0	24.0	0.2	0.1	0.1
III	24–59	Yellowish-brown 10 YR 5/6	4.0	8.9	0.6	1.4±0.28	<0.1	<0.1	77.0	22.7	<0.1	<0.1	<0.1	<0.1
IV	59–61	Red-yellowish-brown 5 YR 4/5	3.6	14.6	0.6	1.5±0.04	<0.1	69.4	27.9	2.6	<0.1	<0.1	<0.1	<0.1
V	61–69	Grey 5 YR 2/1	4.1	16.1	0.6	1.3±0.09	<0.1	13.9	76.3	9.6	<0.1	<0.1	<0.1	0.5
VI	69–78	Light reddish-brown 5 YR 5/5	3.8	18.0	0.8	2.2±0.66	<0.1	18.5	68.5	12.4	0.2	0.2	0.1	0.2
VII	78–83	Reddish-brown 5 YR 4/4	3.7	14.4	1.0	1.6±0.09	0.2	38.7	56.4	4.5	<0.1	<0.1	<0.1	<0.1
VIII	83–95	Yellowish-brown 10 YR 5/5	3.7	17.6	0.9	2.2±1.40	<0.1	1.9	53.7	44.0	0.2	0.1	<0.1	<0.1

Classification was based on the German standard (DIN 4022-1, 1987) with focus on grain sizes and colors (using Munsell rock-color chart, Geological Society of America, 1995).

[a] ww = wet weight.
[b] dw = dry weight.
[c] G = gravel.
[d] cS = coarse sand.
[e] mS = medium sand.
[f] fS = fine sand.
[g] cU = coarse silt.
[h] mU = medium silt.
[i] fU = fine silt.
[j] T = clay.

For determination of the total element content, sediment was digested with concentrated hydrofluoric acid, nitric acid, and perchloric acid at 150–170 °C in a pressure digestion system (DAS, PicoTrace, Bovenden, Germany). The elements were measured in the resulting solutions with inductively coupled plasma-optical emission spectrometry (ICP-OES, Spectroflame P FAV05, Spectro Analytical Instruments, Kleve, Germany, for Fe, Mn); and inductively coupled plasma-mass spectrometry (ICP-MS, PlasmaQuad PQ3-S-Option, VG Elemental, Winsford, UK, for As, Cd, Co, Cr, Cu, Ni, U, Zn).

Binding forms of the elements were determined by sequential extraction of air-dried and sieved (<2 mm) sediment samples using the method of Zeien and Brümmer (1989). The resulting fractions were analyzed with ICP-OES and ICP-MS as described above. The method yielded the following seven fractions, which can be correlated to putative binding forms: mobile (fraction 1), specifically adsorbed (fraction 2), elements bound to manganese oxides (fraction 3), elements bound to organic material (fraction 4), elements bound to amorphous iron oxides (fraction 5), elements bound to crystalline iron oxides (fraction 6), elements bound to the residual fraction, presumably mainly silicates (fraction 7). The extraction method is explained in more detail in Grawunder et al. (2008, this issue).

2.3. Porewater analyses

The pH was measured using a pH-meter pH330 (WTW, Weilheim, Germany) with a micro-pH-electrode (Inlab 423, Mettler-Toledo, Gießen, Germany). Iron (II) was measured after HCl extraction (Reiche et al., 2008) with the phenantroliniumchloride method (Tamura et al., 1974). After the photometric measurement (512 nm, Uvikon 931, Kontron Instruments, Groß-Zimmern, Germany), 100 mL ascorbic acid (10% (w/v)) was added to each sample to reduce iron (III), and the measurement was repeated to obtain the total soluble iron content. Manganese was measured with the formaldoxim method described by Kostka and Nealson (1998). Nitrate was measured as described by Velghe and Claeys (1985). Ammonia was determined using the phenol-hypochlorite method (Harwood and Kühn, 1970). For determination of sulfate the barium method (Tabatabai, 1974) was used. Metals, radionuclides, and total sulfur were measured in the porewater samples after acidification with nitric acid with ICP-OES and ICP-MS as described above. Since this site is highly contaminated with sulfate, total sulfur in the porewater was considered to be sulfate.

2.4. Microbiological analyses

All microbiological analyses were done with fresh sediment samples homogenized by mixing with sterile spatulas. To determine aerobic CO_2-formation rates, 30 g sediment was filled in 150 mL infusion bottles and sealed with butyl rubber stoppers and aluminum screw caps. An overpressure of 120–150 mbar was adjusted by injecting filter-sterilized atmospheric air. Headspace CO_2 concentrations were measured at selective time intervals during 8 h by injecting 100 µL headspace volume into a GC-TCD (Gaschromatograph with thermal conductivity detector, Hewlett Packard 5890, Böblingen, Germany) as described previously (Reiche et al., 2008). Headspace pressure was monitored using a TensioCheck TC 1066 (Tensio-Technik, Geisenheim, Germany). CO_2-formation rates were calculated from the slope of the linear phase of CO_2 increase over time. The measurements were done in triplicate.

Total numbers of microbial cells were determined by microscopic counting after staining with 4′,6-diamidino-2-phenylindole (DAPI) as described by Porter and Feig (1980). Microorganisms were removed from sediment particles with a chemical and mechanical extraction following protocols from Mermillod-Blondin et al. (2001) and Lunau et al. (2005). For separation and fixation 5 mL filter-sterilized extraction solution (5 mM Na-pyrophosphate, 70 mM NaCl, 5 mM EDTA, 10% (v/v) methanol, 4% (v/v) formaldehyde) was added to 1 g sediment, mixed thoroughly and incubated for 30 min in a sonication bath (SONOREX DK 514 BP, 35 kHz, 2 × 450 W, BANDELIN, Berlin, Germany) at 60% intensity. After 30 min, 2 mL of the supernatant was taken and stored at 4 °C until staining. Staining was done following the protocol of Schallenberg et al. (1989). Cells were counted in duplicates with a Zeiss Axioplan Microscope (Jena, Germany) at 1000 × magnification.

To test for heavy metal resistance, aerobic culturable cells were enumerated using a most probable number approach in the absence of additional heavy metals or in the presence of a final concentration of 0.1 mM Cu, and 5.0 mM Ni. Concentrations of Ni and Cu were adjusted to the tolerance range tested for microorganisms from metal-contaminated soils downstream of the former heap Gessenhalde (Schmidt et al., 2005). For cultivation a tryptic soy broth (TSB) medium (550 mg TSB/L) prepared with filter-sterilized groundwater was inoculated with a serially diluted sediment sample. Cycloheximide (50 mg/L) was added to inhibit fungal growth on microtiter plates. For each dilution, 10 replicates were inoculated. The cultures were incubated in the dark at 15 °C and growth was observed visually. The number of culturable cells and significance of differences was estimated using the tables by Alef (1991).

Anaerobic microbial activities were measured in sediment microcosms prepared with 30 g sediment and 60 mL filter-sterilized, anoxic groundwater under a gas flow of sterile Ar. Bottles were sealed with butyl rubber stoppers and aluminum screw caps, put on a horizontal shaker for 1 h, and incubated with an initial overpressure of 950 mbar in the dark at 15 °C. As a control the filter-sterilized groundwater was used without addition of sediment. At selective time intervals, the suspension was sampled with sterile syringes connected to 21 gauge needles. Samples for Fe and ammonium were taken directly from this suspension immediately after sampling. The remaining suspension was centrifuged, and pH, nitrate, manganese, and sulfate were measured in the supernatant. All measurements were done as described above. The formation of CO_2 and methane (CH_4) was measured at selective time intervals. CO_2 was detected as described above. Methane was measured with a GC-FID (Gaschromatograph with flame ionization detector, Hewlett Packard 5890, Böblingen, Germany) as described previously (Küsel and Drake, 1995; Reiche et al., 2008). After 66 days of incubation, yeast extract was added from a sterile solution to yield a final concentration of 0.1% (w/v).

For statistical analysis a one-way analysis of variance (ANOVA) was performed using SPSS 15.0 (SPSS Inc., Chicago, IL, USA). A Bonferroni post-hoc test was used to compare single time points. Values were transformed for statistical tests (log10 (value+1)). The level of significance was 0.05.

3. Results

3.1. Composition of sediment solid phase

Eight different layers were differentiated based on differences in colour and grain size distribution (Table 1). Grain size analyses revealed that the dominating grain sizes below the allochthonic soil material (layers I and II) consisted of coarse and medium sand (Table 1) due to sandy quaternary glacilimnic sediments deposited in the Elsterian glacial period (Eißmann, 1997). The groundwater level fluctuated between 63 and 90 cm depth in 2006. Sediment organic matter content never exceeded 1.1% (w/w). Total nitrogen and sulfur contents were below the detection limit of 0.3% (w/w) in all layers. Carbonate was not detected.

Due to the allochthonic origin of layers I and II, we focused on the deeper layers. The grey layer V and the reddish-brown layer VII were partially cemented indicating enhanced precipitation of iron and manganese minerals. High concentrations of manganese in the solid phase (19 mg/g) were present only in layer V, whereas iron peaked in layers IV, VI, VII, and VIII with highest concentrations of up to 82 mg/g in layer VII (Fig. 2). Accumulations of other metals were detected mainly in sediment layers with elevated iron (IV, VI, VII) or

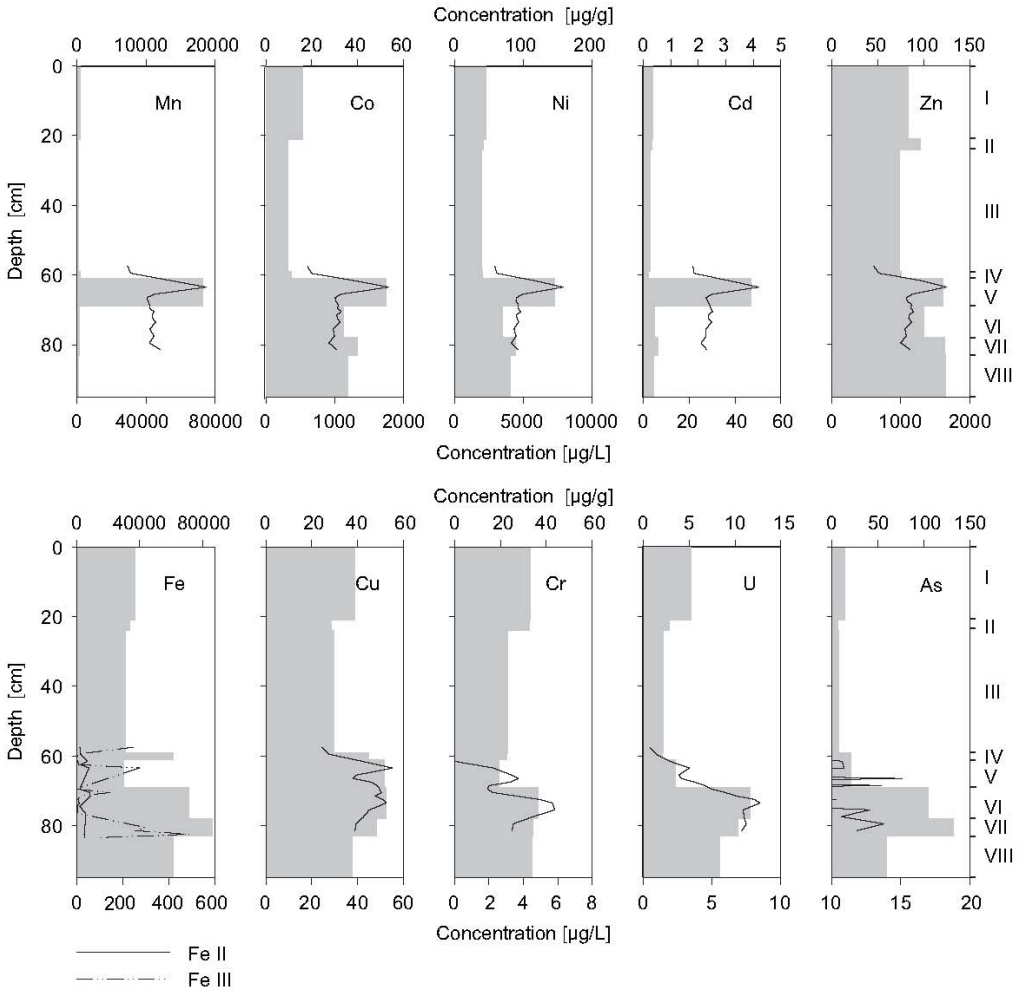

Fig. 2. Metal concentrations of the sediment (filled areas) along the profile (sediment layers I–VIII, localization of sampled sediment layers is given at the right axis) and metal concentrations in the pore water (lines) of the water-saturated sediment layers V–VII collected with a dialysis sampler.

manganese content (V) (Fig. 2). Total concentrations of Cd, Ni, and Co peaked only in the grey sediment layer V. Elevated total concentrations of Cu and Zn were present in both manganese- and iron-rich layers, whereas elevated concentrations of Cr, U, and the metalloid As were detected only in the iron-rich layers. Ni and As reached up to 150 μg/g.

The main component of all samples was quartz. Also alkali feldspars, clay minerals (muscovite, illite, chlorite), and gypsum were identified. In the iron-rich layers IV, VI, VII, and VIII goethite was detected by XRD. In the manganese-rich layer V, also a manganese barium oxide, probably Birnessite, was identified (Matthias Händel, personal communication). The iron and manganese oxides occurred mainly as covers on quartz grains and also as cement between the quartz grains in areas with more dense precipitation.

3.2. Sorbents for heavy metals

When sediment samples of layers III VII were subjected to sequential extractions, highest concentrations of heavy metals were detected mainly in the residual fraction (fraction 7) and in the crystalline iron oxide fraction (fraction 6) (Fig. 3). Especially in the iron-rich layers VI and VII, the importance of crystalline iron oxides increased as sorbents for As, and the metals U, Ni, Zn, Cd, and Co. Amorphous iron oxides (fraction 5) appeared to be less important sorbents for heavy metals. Only in layer IV, concentrations of Cu and Zn were elevated and in layers VI and VII, concentrations of Cr and U were elevated in fraction 5. Thus, retention of heavy metals appeared to be mainly linked to crystalline iron oxides compared to amorphous iron oxides in this sediment.

Organic compounds (fraction 4) appeared to be not important as sorbents for heavy metals, which might be due to the low amount of organic carbon in this sediment. Similarly, only small amounts of heavy metals were present in the manganese oxide fraction (fraction 3), which might be due to the low amount of manganese present in the sediment profile except for the manganese-rich layer V. In layer V, Ni, Co, and Cd reached highest concentrations in fraction 3 and Cu and Zn reached similar concentrations in fraction 3 like in fractions 6 and 7, demonstrating the importance of Mn oxides as sorbents in the grey cemented layer V. Only As, Cr, and U did not show elevated concentrations in fraction 3 in layer V.

Ratios of heavy metal contents of fraction 3 normalized to the total Mn content of sediment layers III, V, and VII varied between 0 and 42×10^{-3} (Fig. 4A). In layer V, the highest ratio was observed for Ni. However, no preference for a specific heavy metal could be observed when all three layers were compared. Although the Mn content of layer V was approximately 70 times higher than in layers III and VII, ratios were still comparable. Ratios of heavy metal contents of fractions 5 and 6 normalized to the total Fe content of sediment layers III, V, and VII (Fig. 4B) were upto 65 times lower compared to ratios mentioned above. For As high As/Fe ratios but not As/Mn ratios were observed, which might be due to the positive surface charge of iron oxides.

The specifically adsorbed fraction (fraction 2) was important as sorbent only for U. 30% and 24% of U of the total uranium concentration were present in fraction 2 in layers VI and VII. According to Zeien and Brümmer (1989) fraction 2 includes specifically adsorbed metals, metals occluded near the surface, metals bound in carbonates, and organometallic complexes. Since no carbonates were present and the organic carbon content was low, U might be mainly adsorbed or occluded near the surface. Highest concentrations of Ni and Cd in the mobile fraction (fraction 1) occurred in layer V.

3.3. Porewater composition

Porewater was sampled in the water-saturated layers V VII. The pH of the water varied between 4 and 5 with a slight increase below 75 cm (Fig. 5). Concentrations of nitrate varied between 20 and 200 μM, but showed no decreasing trend with increasing depth. Small concentrations of ammonia (< 30 μM, data not shown) were present. Average concentrations of sulfur in the porewater were high (10 mM) and reached even 30 mM in 63 cm depth in sediment layer V (Fig. 5). The heavy metals Mn, Ni, Co, Zn, Cu, and Cd also peaked in the porewater obtained in this depth (Fig. 2). Elevated concentrations of Cu were also detected in layers VI and VII. Concentrations of U and Cr peaked in approximately 74 cm depth (layer VI). Concentrations of As were near the detection limit (10 μg/L) in the sediment profile. Concentrations of Fe(II) were low, but Fe(III) reached up to 400 μM in layer VII (Fig. 2).

3.4. Microbial abundance and activity

Basal sediment respiration rates were low and approximated 9 and 5 nmol g^{-1} [wet weight (ww)] h^{-1} in layers I and II, respectively, and 2 nmol g^{-1} [ww] h^{-1} in layers III VIII (Table 1). MPN numbers of culturable cells quantified from cultures inoculated with material from different layers reached 10^5 10^6 cells/g sediment (ww) for layers I, II, and V, but only 10^3 10^4 cells/g sediment (ww) for layer VII (Table 2). The presence of 5 mM Ni inhibited microbial growth only in cultures inoculated with material from layer VII. The presence of 0.1 mM Cu inhibited slightly the microbial growth in cultures inoculated with material from layer II. Total

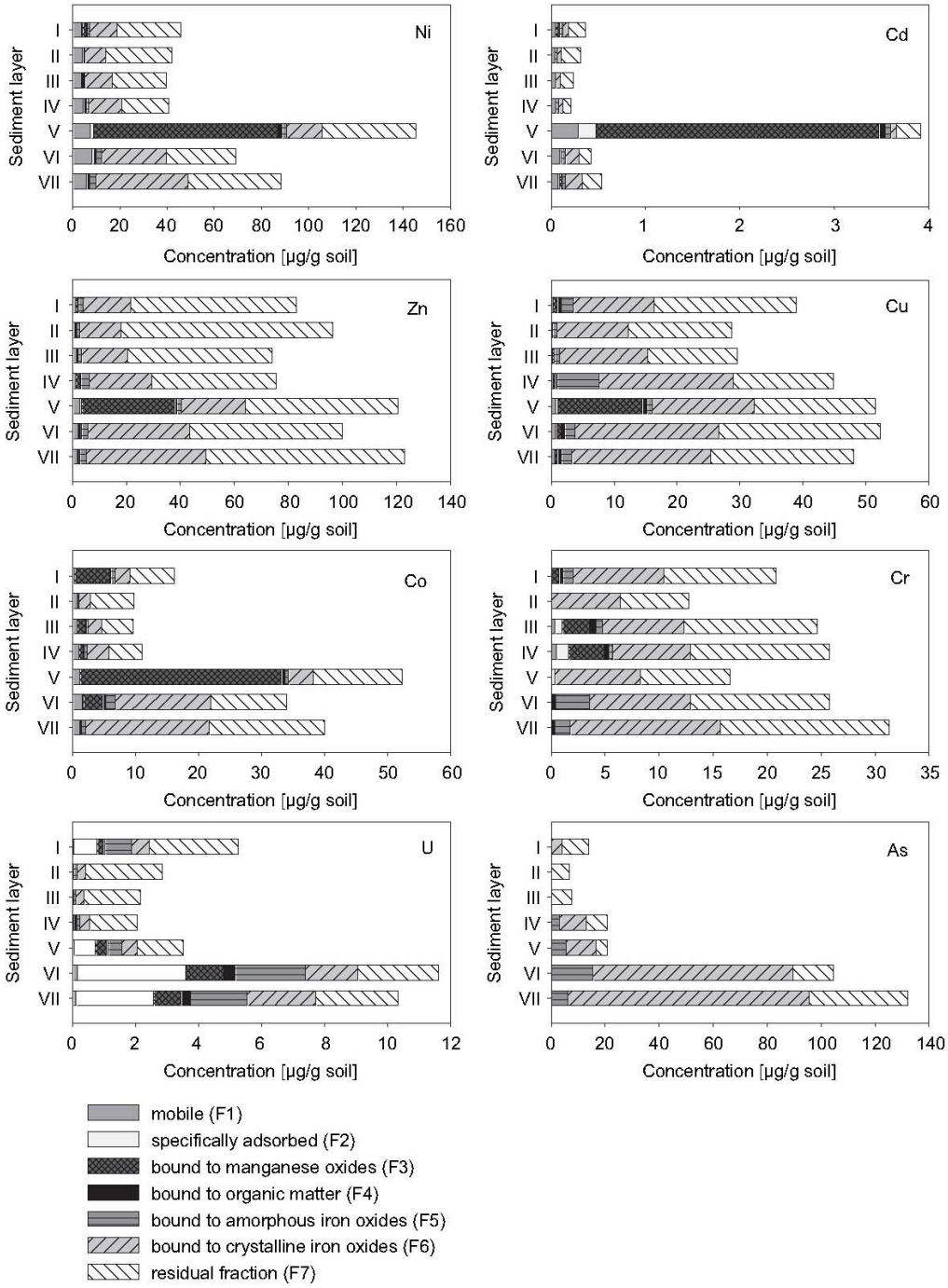

Fig. 3. Concentrations of selected metals in fractions obtained by sequential extraction from sediment layers I–VII.

microbial cell numbers approximated 10^6 cells/g sediment (ww) in layers III VII (data not shown).

Anaerobic reductive microbial activities could not be detected during 66 days when sediments of layers V and VII were incubated under anoxic conditions. After addition of yeast extract (day 66) a significant decrease in the concentration of nitrate was observed in microcosms of layer VII ($F_4 = 9.621$, $p = 0.002$), but not in the controls without sediment ($F_4 = 1.114$, $p = 0.402$, Fig. 6). The concentration of Fe(II) increased after 70

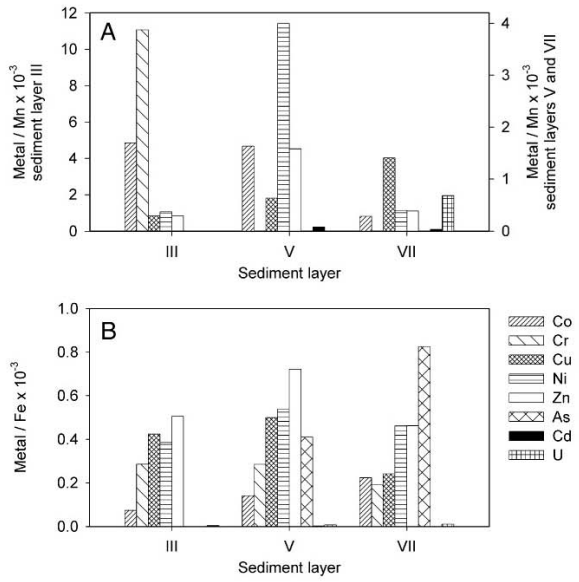

Fig. 4. Molar ratios of heavy metals obtained in fraction 3 to the total Mn content (A) and molar ratios of heavy metals obtained in fractions 5 and 6 to the total Fe content (B) in layers III, V, and VII. Note the different scales in A for III (left axis), and V, VII (right axis).

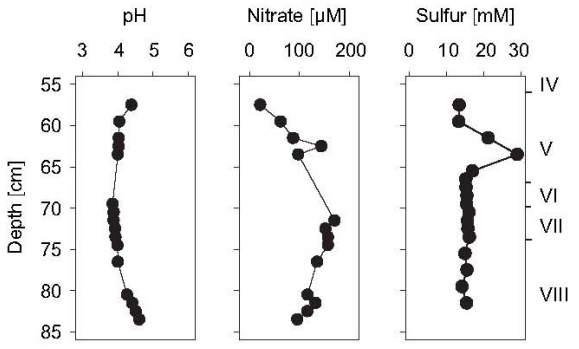

Fig. 5. Porewater profiles of pH and concentrations of nitrate and sulfur of the water-saturated layers V–VII collected with a dialysis sampler. Depth of sediment layers for soil water sampling (right axis) differed from solid phase sampling because of inhomogeneities in the sediment.

days of incubation in yeast extract amended microcosms of layer VII ($F_4 = 10.186$, $p = 0.001$), but concentrations of Mn did not increase. Although significant differences in sulfate concentrations were detected in layers V and VII microcosms, no significant differences occurred between the first and last time points measured after the addition of yeast extract (layer V: $p = 0.3340$, layer VII: $p = 0.2386$), indicating that there was no real change in sulfate concentrations. In contrast, the addition of yeast extract did not significantly stimulate nitrate-consuming, Fe(II)-forming, Mn-mobilizing, and sulfate-consuming activities in microcosms of layer V. In summary, these results indicated the onset of nitrate- and Fe(III)-reduction only in layer VII after addition of a carbon and nitrogen source.

4. Discussion

4.1. Geochemical barriers

Due to the substantial accumulation of heavy metals in layers V and VII, these layers can be described as geochemical barriers (Perel'man, 1967; Huang and Gong, 2005). The concentrations of Cd, Co, and Ni in the grey manganese-rich layer V exceeded their average corresponding concentrations in layers I IV by a factor of 7, 4, and 3, respectively. In the reddish-brown iron-rich layer VII, the concentrations of As, U, Co, and Ni exceeded their average corresponding concentrations in layers I IV by a factor of 13, 4, 3, and 2, respectively. These differences could not be attributed to differences in grain size distribution. The local accumulation of heavy metals seemed to be accompanied by mineralization processes. Iron and manganese oxides occurred mainly as coverage of quartz grains and as cement between the quartz grains, which might further close the porous sediment matrix. Although agglutination of particles occurred in layer VII apparently due to processes driven by capillary transport within an oxygen dominated environment, the porosity was not reduced sufficiently to use the term hardpan as defined by Graupner et al. (2007) like in an earlier study (Carlsson and Büchel, 2005). Similarly, both barriers cannot be termed as cemented layers (Graupner et al., 2007), because biogeochemical parameters of the porewater did not reveal reduced conditions in deeper water-saturated layers or other variations in master geochemical variables.

It is not clear if the formation of the geochemical barriers is still an on-going process. Their formation seemed to be initiated by a local leakage of radionuclides and other heavy metals through the drainage layer (1 m depth) and the barrier loam layer (0.6 m depth) at the base of the heap Gessenhalde during leaching of the black shale material (Grawunder et al., 2008, this issue). Although the heap material was removed in 1993 to the open mine Lichtenberg and the base layers were taken off to a maximum depth of about 10 m, mineralization and heavy metal retention near the groundwater table might be still fuelled by capillary rise of contaminated groundwater.

Previous investigations demonstrated a spatially heterogeneous, residual contamination of the glacial sediments of the test site Gessenwiese where metals only locally reach values above reference values

Table 2. MPN estimates of aerobic microorganisms cultured in pH 5 groundwater medium in the presence or absence of supplemented heavy metals.

Medium ± metals	MPN g (ww sediment)$^{-1}$ (95% confidence limit)[a]			
	Layer I	Layer II	Layer V	Layer VII
Without metals	2×10^5 (1.1–2.6)^{A}	1.8×10^6 (1.0–2.4)^{A}	2.4×10^5 (1.4–3.2)^{A,B}	2×10^4 (1.1–2.6)^{A}
With Ni (5 mM)	5×10^5 (2.8–6.6)^{A}	Contaminated	5×10^5 (2.8–6.6)^{A}	4.6×10^3 (2.6–6.1)^{B}
With Cu (0.1 mM)	2.8×10^5 (1.6–3.7)^{A}	5.2×10^5 (3.0–6.9)^{B}	1.4×10^5 (0.8–1.8)^{B}	3.4×10^4 (1.9–4.5)^{A}

The values were estimated from 10 replicates.

^{A,B}Significant differences of the MPN values between the different treatments for each sediment layer. Significance is given when the ratio between MPNs (cells g^{-1} (ww sediment)) is above 3.28 (Alef, 1991).

[a]95% confidence limit with the same exponent as the mean.

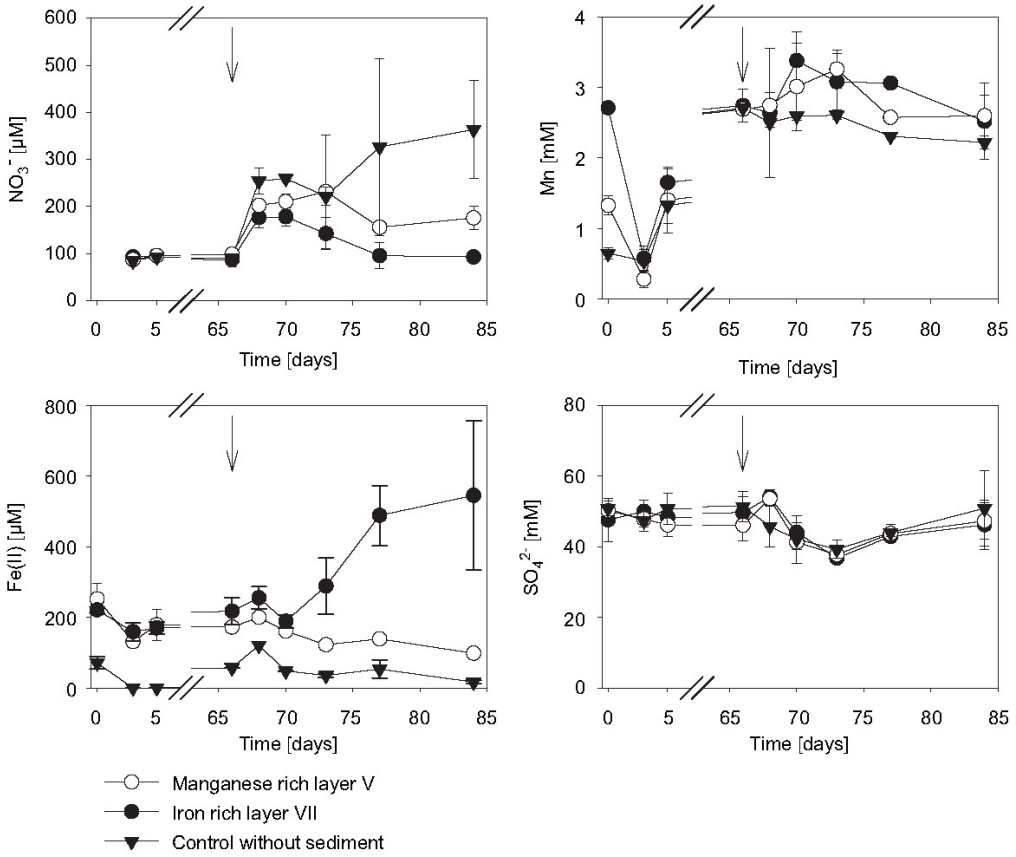

Fig. 6. Time-dependent concentrations of nitrate, Fe(II), soluble manganese, and sulfate in anoxic microcosm experiments with sediment of layers V and VII in comparison with a control without sediment. The time point of addition of ~0.1% (w/v) yeast extract after 66 days of incubation is indicated by an arrow. The values represent means of triplicates ± standard deviation.

(Grawunder et al., 2008, this issue). Heavy metal concentrations in layers I IV of this study were comparable to concentrations measured at other sites of the test site Gessenwiese (Grawunder et al., 2008, this issue). Despite the remediation activities the values of As, Cd, Cr, Cu, Mn, Ni, U, and Zn in the entire profile were elevated compared to background values for a soil derived from periglacial sediments like a sandy loam in Saxony (available at http://www.umwelt.sachsen.de/de/wu/umwelt/lfug/lfug-internet/boden_1969.html; accessed 2008-01-28). With the exception of the metalloid As element concentrations in all sediment layers were below

values from the German regulation for soil in recreational areas (BBodSchV, 1999). However, not only Ni, but also As and Cd concentrations in the porewater exceeded values of the German Drinking Water Ordinance (TrinkwV, 2001). The spatially heterogeneous leakage through the barrier loam layer might be responsible for the patchy occurrence of geochemical barriers at the test site and for spatially different heavy metal concentrations. For example, the concentrations of As (132.2 μg/g) and U (10.4 μg/g) in layer VII of this study were much lower than the concentrations of As (295.5 μg/g) and U (84.6 μg/g) in the hardpan described by Carlsson and Büchel (2005).

Sequential extractions performed with samples of two excavations (L and Mo, Fig. 1) show high amounts of metals (Co, Ni, and U) in the mobile fraction 1 suggesting a further downstream movement of these residual contaminations also to pristine areas due to the low pH of the groundwater (Grawunder et al., 2008, this issue). Thus, the long-term stability of the geochemical barriers causing temporary natural attenuation of toxic compounds will be important for the whole catchment.

4.2. Metal retention

Crystalline iron oxides, mainly goethite, and not amorphous iron oxides served as important sorbents for As, Zn, Ni, Cu, Cr, Co, U, and Cd in the iron-rich layers VI and VII. These iron-rich layers are similar to the hardpan described by Carlsson and Büchel (2005). However, Ni, Cd, and Co reached even higher concentrations in fraction 3 in the grey manganese-rich layer V pointing to the predominance of manganese oxides as sorbents for heavy metals similar to other studies (Latrille et al., 2001; Palumbo et al., 2001; Liu et al., 2002; Cornu et al., 2005; Covelo et al., 2007; Dong et al., 2007). The molar ratios of heavy metals of fraction 3 to the Mn content were 2 10 times higher than the ratios of heavy metals of fractions 5 and 6 normalized to the total Fe content. The ratios were not affected by the high amount of Mn present in layer V. Thus, during the mineralization of Mn oxides similar amounts of heavy metals appeared to be co-precipitated or sorbed to the secondary minerals in all layers. This constant ratio is in contrast to the decreasing ratios detected in Fe Mn-nodules where lower amounts of trace elements adsorb or co-precipitate during growth of the nodule (Palumbo et al., 2001).

Approximately 8, 6, 3, or 3 times more of the metals Ni, Co, Cu, or Zn, respectively, were observed per mol Mn present in layer V than per mol Fe present in layer VII. Thus, the manganese oxide barrier appeared to be more effective in metal retention than the iron oxide barrier. The presence of manganese oxides, e.g. Birnessite (Matthias Händel, personal communication), and their dominant role as metal scavenger in this low pH sediment were surprising, because manganese oxides are not thought to form below pH 7 (Lee et al., 2002). Minerals of the braunite group are known to incorporate As and Zn in the mineral (Rösler, 1991). However, in our study As was enriched in the crystalline iron oxide fraction similar to results of cemented layers subjected to sequential extractions (Graupner et al., 2007).

4.3. Microbiological activities in geochemical barriers

In general, the microbial abundance and activities in the sediment profile were low, which might be due to the low organic carbon content of the sediment or to toxic effects of heavy metals. The 5 or 2 times higher basal respiration rates of the cover layers I and II compared to the deeper sediment layers might be due to the effect of the sparse vegetation rooting in these layers releasing easily available carbon sources. Maximum total cell numbers approximated only 10^6 cells/g dry weight sediment compared to 10^7 10^{10} cells/g dry weight soil or sediment reported in other studies (Christensen et al., 1999; Taylor et al., 2002; Braun et al., 2006). Numbers of cells cultured under oxic conditions in pH 5 groundwater media yielded often 10% of the total cell numbers counted after DAPI staining, which is much higher than the usually cultured 0.1% of total prokaryotic cells (Hill et al., 2000). The high percentage suggested that the growth conditions used were well suited for the indigenous microbial communities.

The concentrations of Zn, Ni, and Cr in the porewater and in the bioavailable fractions I and II reached a concentration range where toxic effects on microorganisms were observed (Scheffer and Schachtschabel, 2002). However, the addition of Ni (5 mM which corresponds to 293 mg/L) or Cu (0.1 mM which corresponds to 6 mg/L) in a concentration range tested for heavy metal tolerance of microorganisms present at an adjacent site (Schmidt et al., 2005) had only small inhibitory effects in cultures inoculated with material from layers VII or II, respectively. These inhibitions could not be correlated with *in situ* metal concentrations in the soil profile. Thus, the microbial communities appeared to be well adapted to the presence of heavy metals.

Geochemical parameters measured in the porewater profile showed that nitrate or sulfate were not depleted with increasing sediment depth and that Fe(III) still peaked in layer VII. Although the Eh was not measured, the presence of nitrate and Fe(III) indicated oxic conditions throughout the sediment profile. In addition, no nitrate-, manganese-, Fe(III)-, or sulfate-reducing activities could be observed during 66 days of anoxic sediment incubation, suggesting that metals will not be released and uranium will not be reduced by anaerobic

microorganisms *in situ*. However, microbial activity in contaminated subsurface environments is often limited by carbon availability (Edwards et al., 2007). Thus, the addition of yeast extract as carbon source stimulated the reduction of nitrate and Fe(III) in microcosm experiments of layer VII showing the potential ability of the indigenous microbial community for these reductive processes. However, these anaerobic processes are not likely to occur *in situ* due to the low organic carbon sources available in the soil. After development of a more dense vegetation in the next decades, nitrate- and Fe(III)-reducing activities might be initiated and affect metal mobility.

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Impact of biostimulated redox processes on metal dynamics in an iron-rich creek soil of a former uranium mining area

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Abstract

Understanding the dynamics of metals and radionuclides in soil environments is necessary for evaluating risks to pristine sites. An iron-rich creek soil of a former uranium-mining district (Ronneburg, Germany) showed high porewater concentrations of heavy metals and radionuclides. Thus, this study aims to i) evaluate metal dynamics during terminal electron accepting processes (TEAPs) and ii) characterize active microbial populations in biostimulated soil microcosms using a stable isotope probing (SIP) approach. In biostimulated soil slurries, concentrations of soluble Co, Ni, Zn, As, and unexpectedly U increased during Fe(III)-reduction. This suggests that there was a release of sorbed metals and As during reductive dissolution of Fe(III)-oxides. Subsequent sulfate-reduction was concurrent with a decrease of U, Co, Ni, and Zn concentrations. The relative contribution of U(IV) in the solid phase changed from 18.5 to 88.7% after incubation. The active Fe(III)-reducing population was dominated by *δ-Proteobacteria* (*Geobacter*) in ^{13}C-ethanol amended microcosms. A more diverse community was present in ^{13}C-lactate amended microcosms including taxa related to *Acidobacteria*, *Firmicutes*, *δ-Proteobacteria*, and *β-Proteobacteria*. Our results suggested that biostimulated Fe(III)-reducing communities facilitated the release of metals including U to groundwater which is in contrast to other studies.

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Impact of Biostimulated Redox Processes on Metal Dynamics in an Iron-Rich Creek Soil of a Former Uranium Mining Area

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Understanding the dynamics of metals and radionuclides in soil environments is necessary for evaluating risks to pristine sites. An iron-rich creek soil of a former uranium-mining district (Ronneburg, Germany) showed high porewater concentrations of heavy metals and radionuclides. Thus, this study aims to (i) evaluate metal dynamics during terminal electron accepting processes (TEAPs) and (ii) characterize active microbial populations in biostimulated soil microcosms using a stable isotope probing (SIP) approach. In biostimulated soil slurries, concentrations of soluble Co, Ni, Zn, As, and unexpectedly U increased during Fe(III)-reduction. This suggests that there was a release of sorbed metals and As during reductive dissolution of Fe(III)-oxides. Subsequent sulfate-reduction was concurrent with a decrease of U, Co, Ni, and Zn concentrations. The relative contribution of U(IV) in the solid phase changed from 18.5 to 88.7% after incubation. The active Fe(III)-reducing population was dominated by *δ-Proteobacteria* (*Geobacter*) in ^{13}C-ethanol amended microcosms. A more diverse community was present in ^{13}C-lactate amended microcosms including taxa related to *Acidobacteria*, *Firmicutes*, *δ-Proteobacteria*, and *β-Proteobacteria*. Our results suggested that biostimulated Fe(III)-reducing communities facilitated the release of metals including U to groundwater which is in contrast to other studies.

Introduction

Natural attenuation, enhanced or mediated by microbial activities, can immobilize heavy metals and radionuclides and prevent their transport by different mechanisms, such as sorption and precipitation processes, and redox state transformations (*1, 2*). Metal- and sulfate-reducing microorganisms can immobilize uranium (U) by direct enzymatic reduction to an insoluble and less mobile form (*3, 4*), or indirectly via production of reducing agents, e.g., Fe(II) and sulfide (*5, 6*). Reductive immobilization of U was observed among a phylogenetically diverse assemblage of respiratory and fermentative microorganisms. In particular, members of the Fe(III)-reducing *Geobacteraceae* family (*δ-Proteobacteria*) have been demonstrated to be important for bioremediation at contaminated subsurface sites (*7, 8, 2*). However, metal-reducing microorganisms also can cause metal release by reductive dissolution of iron- or manganese-oxides which are important metal scavengers (*9, 10*).

In the most productive mining district of the former German Democratic Republic, Ronneburg (Thuringia, Germany), uranium mining caused severe environmental contamination (*11*). The creek Gessenbach was one of the main drainage systems for the former mining sites (*12*). In contrast to other well investigated U contaminated sites (Rifle, CO and Oak Ridge, TN), the remediated Ronneburg mining district contains high concentrations of a variety of heavy metals, e.g., Cu, Ni, Cd, As, Zn, Pb, Cr, and Co, which raises the question how multiple metal contaminants are effected by microbial activities under changing redox conditions. This study aims to link microbial activities with metal dynamics in an iron-rich creek soil at the bank of the Gessenbach in biostimulated microcosms incubated first under anoxic conditions and then during reoxidation. The active microbial populations during terminal electron accepting processes (TEAPs) in these microcosms were identified with stable isotope probing (SIP).

Materials and Methods

Study Site and Sampling. The study site is the bank of the creek Gessenbach downstream of the former mining sites near Ronneburg (Thuringia, Germany, location: E 4510121, N 5635807, Gauss/Krueger Potsdam coordinate system), which receives contaminated groundwater. Addition of HCl did not indicate the presence of carbonate in the soil. The groundwater influenced oxidized horizon Btlc contained the highest concentrations of U, Cu, Zn, Ni, Pb, and As in the solid phase that approximated 1.57, 644, 466, 205, 191, and 85 $\mu g\ g^{-1}$, respectively (*13*). The organic carbon, Fe, and Mn contents were 6%, 65 and 0.4 mg g^{-1}, respectively.

Soil−water was sampled monthly in ~10 cm depth intervals from June to November 2007 using Rhizons according to ref *14*. Btlc soil samples for microcosm experiments were taken in May and June 2007 (electron donor experiments), June 2007 (SIP experiment), and April 2008 (metal dynamics experiment) to ensure fresh material for each experiment. Soil was obtained from different sites of the outcrop, pooled, collected in plastic bags, and stored at 15 °C overnight.

Microcosm Experiments. Twenty or 80 g of fresh weight soil (41−47% water content) was placed into sterile 150- or 500-mL incubation flasks, respectively, under a continuous flow of sterile argon and mixed with either 80 or 320 mL of water (amended with 50 μM nitrate, 70 μM ammonium, pH 5.5, according to soil−water concentrations). Flasks were closed, shaken for 1 h, and incubated in the dark at 15 °C with an initial overpressure of 800 mbar. Triplicate microcosms were amended with 10 mM ethanol, 10 mM lactate, 10 mM acetate, 5 mM glucose, or left unamended, respectively. For the SIP experiment ^{13}C-labeled carbon sources (>99 atom % ^{13}C; Cambridge Isotopes, USA) were used. For the metal dynamics experiment, sterile (autoclaved) unamended controls were also prepared. For the reoxidation

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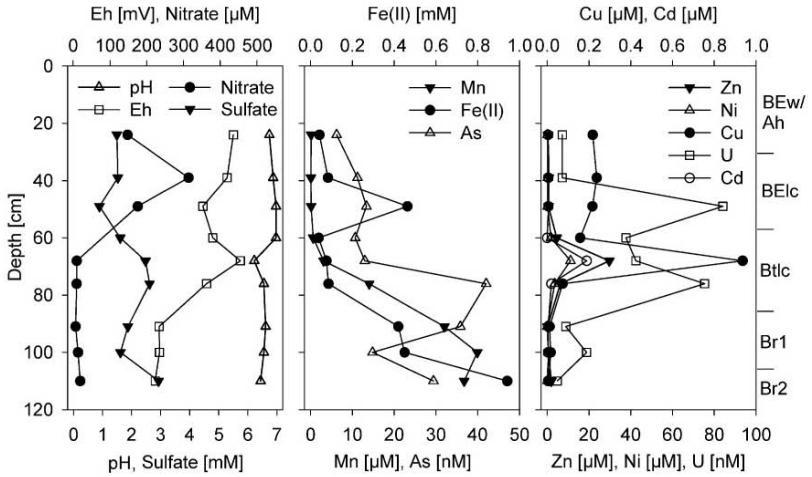

FIGURE 1. Geochemistry of the pore water in the soil profile at the bank of the contaminated creek Gessenbach in August 2007. The corresponding soil horizons are given at the right side of the graphs.

experiment, microcosms of the metal dynamics experiment were opened, closed with sterile cotton stoppers, and incubated on a horizontal shaker. Samples for cultivation-independent community characterization were collected and pooled from each replicate microcosm, centrifuged at 2300*g* for 3 min, and the pellets were frozen at −20 °C until DNA extraction. Procedures used for DNA extraction, separation of ^{13}C- and ^{12}C-labeled DNA, SSU rRNA gene PCR amplification, and terminal restriction fragment length polymorphism (TRFLP) analysis are described in the Supporting Information.

Analytical Techniques. Soil samples for total Fe and Fe(II) determination were extracted in oxalate (0.2 M ammonium oxalate, 0.2 M oxalic acid, pH 2.5) for 4 h in the dark, shaking, as previously described (*15*), and Fe was measured with the phenanthroline method (*16*). Nitrate, ammonium, sulfate, Fe(II), and Fe(III) in the soil solution were measured as described elsewhere (*17*−*21*). Metals and As were measured in acidified, 0.45 µM filtered samples using ICP-MS (inductively coupled plasma−mass spectrometry, X-Series II, Quadrupol ICP-MS, Fa. Thermo Electron, Bremen, Germany). In the microcosm experiments, pH and Eh were measured in the soil suspension. Then, soil suspensions were centrifuged at 2300*g* for 3 min, and the supernatant was analyzed for nitrate and sulfate using ion chromatography (*14*). Iron(II) and total Fe [Fe(II) and Fe(III)] in the soil suspensions were measured after extraction in 0.5 N HCl for 1 h (*20*). Carbon concentrations were determined with high-performance liquid chromatography (*14*).

X-ray Absorption Spectroscopy. X-ray Absorption Near-Edge Structure (XANES) and Extended X-ray Absorption Fine-Structure (EXAFS) spectra were collected at the Rossendorf Beamline at the European Synchrotron Radiation Facility. Btlc soil samples and microcosm solid phase material from triplicate lactate microcosms sampled at the end of incubation were placed on a SH 01 B polyethylene sample holder under anoxic conditions. Additional details are described in the Supporting Information.

Results

Biogeochemistry of the Soil Solution. The geochemistry of the soil solution was comparable at all sampling times. Nitrate was present only in upper horizons (<320 µM) (Figure 1A). Eh declined from 450 to 220 mV, and concentrations of Mn and Fe(II) increased with increasing soil depth (Figure 1B). High sulfate concentrations were observed over the whole soil profile. Concentrations of Cd, Zn, Ni, Cu, and Co peaked in soil solution of horizon Btlc (Figure 1C), and average U concentrations reached 30−80 nM in the BElc and Btlc horizons.

Metal Dynamics under Reducing Conditions. Although the total iron content approximated 323 ± 66 mM with 94% as Fe(III), low to negligible Fe(II) formation rates (≤1.2 µmol g^{-1} d^{-1}) were observed in unamended anoxic soil microcosms. Amendments with lactate or ethanol enhanced rates after a lag phase of 8 days to 3.6 or 4.4 µmol g^{-1} d^{-1}, respectively, whereas amendments with acetate or glucose had no stimulatory effect. Thus, dynamics of TEAPs and soluble metal and As concentrations were studied in lactate (Figure 2) or ethanol (data not shown) stimulated microcosms. Lactate and ethanol supplemented microcosms were also used to identify the active microbial communities. The pattern and kinetics of TEAPs and supplemental carbon consumption were similar in all studies. Nitrate was completely consumed within 2 days (data not shown) followed by an increase in soluble Mn concentrations (Figure 2). Fe(II) concentrations in lactate microcosms increased after 6 days paralleling Mn-reduction accompanied by an increase in soluble metal and As concentrations. Co and Ni concentrations increased immediately, whereas U was released after the onset of Fe(III)-reduction (9 days). Sulfate increased from 0.4 to 0.8 mM during the initial incubation period and decreased after 18 days, paralleling the decrease of Co, Ni, and U. In unamended controls, little to no change in metal or As concentrations were observed and autoclaved controls showed no change. Microcosms amended with ethanol showed patterns similar to those of lactate microcosms. TEAPs observed explained 30−50% of carbon consumption.

XANES. The increase of soluble U under Fe(III)-reducing conditions was unexpected. X-ray absorption near-edge region (up to 17.25 KeV) of the samples fitted with a linear combination fitting approach using a U(IV) and U(VI) component (data not shown) showed that the Btlc soil prior to incubation predominantly contained U(VI) species (81.5%). Soil after anoxic incubation was enriched in reduced U(IV) (88.7%). The linear combination approach provided excellent fits to the data and the obtained values were correct to ±1%. A comparison with a reference spectrum of UO_2 showed a lack of features in the post-edge part of the incubated soil spectrum, suggesting that U(IV) in the incubated soil did not occur in a crystalline form similar to UO_2, but was more likely to be present as a sorbed complex (Figure S1).

Metal Dynamics under Oxidizing Conditions. During the reoxidation experiment, Mn concentrations decreased rapidly within 3 days in the biostimulated microcosms (Figure 3). Concurrently, there was a rapid increase in U concentrations and a rapid decrease in Co and As. Fe(II) concentrations decreased slowly parallel to the increase in

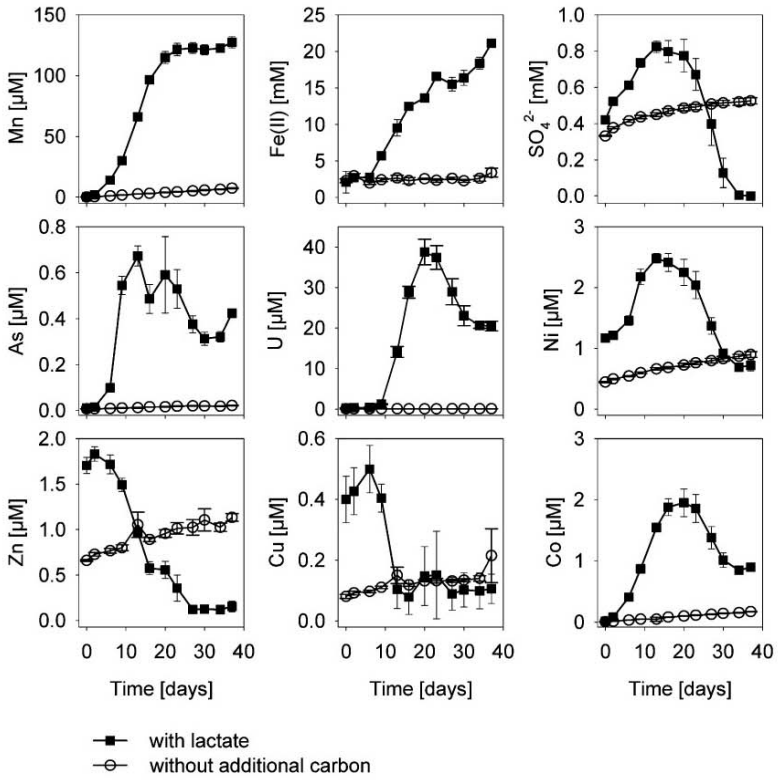

FIGURE 2. Dynamics of Fe(II), soluble Mn, sulfate, and soluble metal concentrations (mean of triplicates ± standard deviations) in anoxic soil microcosms amended with lactate and in controls without additional carbon.

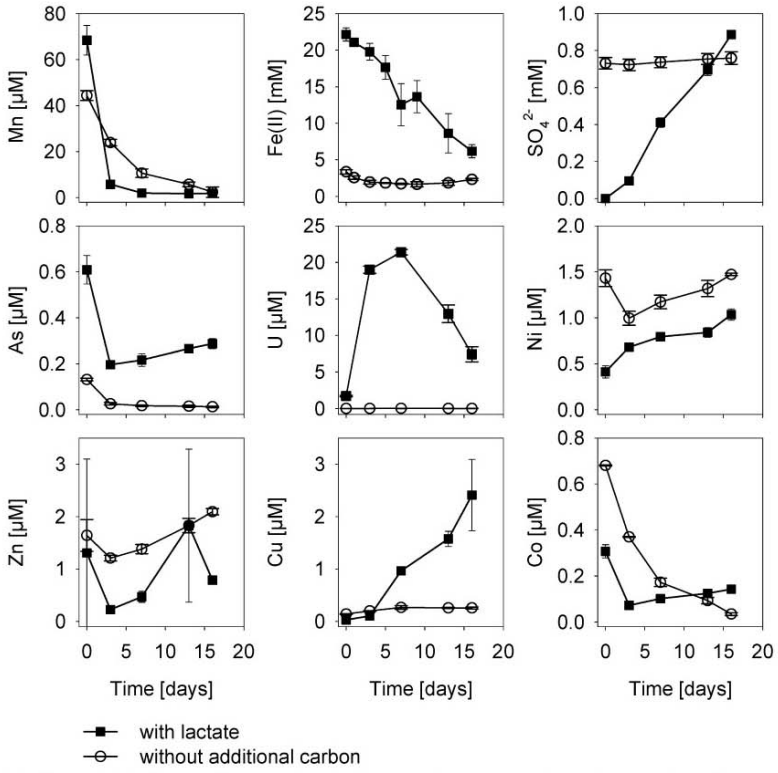

FIGURE 3. Dynamics of Fe(II), soluble Mn, sulfate, and soluble metal concentrations (mean of triplicates ± standard deviations) during reoxidation of formerly reduced soil microcosms amended with lactate at the beginning of the reduction and in controls without additional carbon.

sulfate. After 8 days of reoxidation, U concentrations decreased again.

Characterization of Active Microbial Communities in Anoxic Microcosms. With the next microcosms experiments,

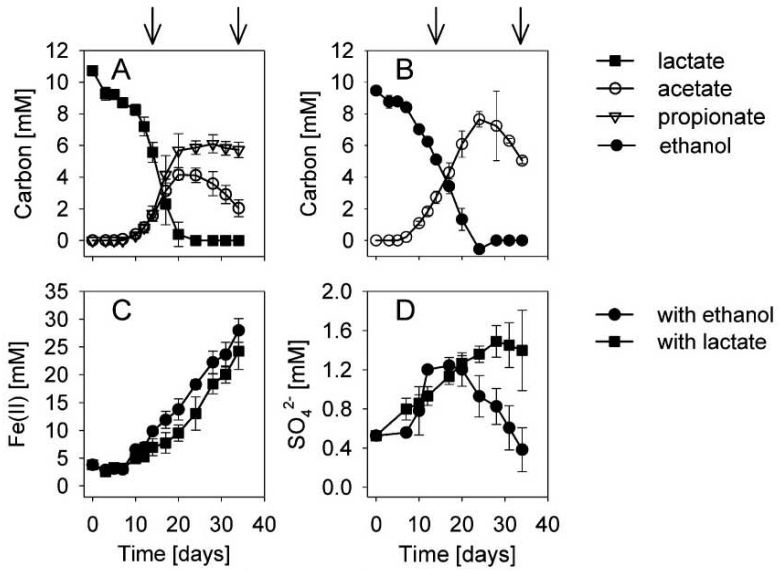

FIGURE 4. Consumption of ^{13}C-lactate (A) or ^{13}C-ethanol (B) in the SIP experiment soil microcosms during Fe(III)- (C) and sulfate-reduction (D) (mean of triplicates ± standard deviations). Arrows indicate time of sampling for SIP.

we wanted to identify the active biostimulated microorganisms with ^{13}C-labeled carbon sources during the periods of Fe(III)- and sulfate-reduction which paralleled changes in heavy metal concentrations. SIP and community characterization were performed at days 14 and 34 corresponding to ^{13}C-carbon usage and TEAPs (Figure 4). Lactate was consumed within 20 days yielding propionate and acetate (Figure 4A) and consumption was linked to Fe(II) formation (Figure 4C). After 20 days, acetate concentrations declined in parallel to an increase in Fe(II) that was followed by a slight decrease of sulfate after 28 days (Figure 4D). Ethanol was completely consumed within 24 days and yielded acetate in parallel to an increase of Fe(II) (Figure 4B and C). After 24 days, acetate declined from 8 to 5 mM in parallel to an Fe(II) increase and a decrease in sulfate from 1.2 to 0.4 mM (Figure 4D). Assuming an 8:1 ratio for acetate oxidation and Fe(III)-reduction and a 1:1 ratio for acetate oxidation and sulfate-reduction, both processes appeared to be stimulated by acetate.

DNA was detected in gradient fractions with buoyant densities ranging from 1.52 to 1.62 g mL^{-1} (Figure S2). The highest density DNA fractions represented the microbial community with the highest ^{13}C incorporation. Comparison of TRFLP patterns generated for each of the DNA fractions revealed a distinct clustering of the heavy fractions only in the ^{13}C-lactate treatment (Figure S3). A total of 40 or 48 clones were screened, yielding coverage of 0.51 or 0.19 for the ethanol or lactate treatments, respectively. The community of the ^{13}C-lactate treatment was dominated by members of the *Acidobacteria*, whereas, the ^{13}C-ethanol treatment was dominated by members of the *δ-Proteobacteria* (Table S1). Additional clones were related to the *Acidobacteria* and *β-Proteobacteria* in the ethanol or *β-Proteobacteria* and *Firmicutes* in the lactate treatments. TRFs of phylotypes were determined by *in silico* and *in vitro* cutting of representative clones and compared to peaks in the TRFLP patterns (Figure S4, Table S1).

TRFs of *Geobacter*-related clones dominated TRFLP patterns from ^{13}C-enriched fractions of the ethanol treatment (days 14 and 34). *Acidobacteria, Geothrix fermentans* (phylotypes 1OE20, 2OE14) and *Firmicutes, Desulfosporosinus* (phylotype 1OE44) TRFs were detected at a lower abundance at 14 days (Figure S4). After 34 days the abundance of *Acidobacteria* and *Firmicutes* increased in the ^{13}C-enriched and intermediate fractions. After 34 days of incubation, a TRF corresponding to a *δ-Proteobacteria*-phylotype with 87% identity to *Pelobacter* was observed in the active DNA fractions indicating a shift in the microbial community over time (Figure S4, Table S1).

In the TRFLP patterns of the ^{13}C-lactate treatment, TRFs corresponding to clones belonging to the *Acidobacteria, Firmicutes,* and *δ-Proteobacteria* were present in similar abundance at 14 days (Figure S4, Table S1). In the clone library, *Acidobacteria* and *Firmicutes* were abundant, but in contrast, *δ-Proteobacteria* had low abundance. *β-Proteobacteria* TRFs, related to *Dechloromonas* and *Janthinobacterium,* were in low abundance in the ^{13}C-lactate treatment (Figure S4). After 34 days, *Acidobacteria* were the most abundant TRFs in the ^{13}C-enriched and intermediate fractions of the lactate treatment, whereas, the abundance of *Firmicutes, β-Proteobacteria,* and *δ-Proteobacteria* decreased (Figure S4).

Discussion

Metal Dynamics during Reductive and Oxidative Periods in Soil Microcosms. This study linked biostimulated Fe(III)-reduction with the release of heavy metals from contaminated creek soil and identified the biostimulated, active microbial communities. Despite the high amount of Fe(III) and organic carbon content (6%) present in the Btlc horizon, this soil showed low to negligible Fe(III)-reduction without supplemental carbon. However, biostimulated Fe(II) formation rates were at least three times higher compared to other studies (*22, 23*). In those studies, ethanol and glucose but not lactate had biostimulatory effects.

The peak of heavy metal concentrations in soil porewater of the Btlc horizon was corroborated with sequential extraction data that demonstrated the presence of high proportions of these metals in the water-soluble or exchangeable fraction ((*13*), and data not shown). Similarly, high proportions of metals were present in the amorphous Fe(III)-oxide fraction, while Mn-oxides were of minor importance. Thus, the reductive dissolution of Fe(III)-oxides might have caused the release of adsorbed or coprecipitated metals (*9, 10, 24*). Concentrations of dissolved Cd and Cu increased prior to Fe(III)-reduction suggesting a release from Mn-oxides. In contrast, Co, Ni, Zn, and As were released during both processes. Differences in metal dynamics could be due to different metal bonding strengths to Fe(III)- or Mn-oxides. Similarly, the increase of sulfate during the beginning of incubation might be due to the release of sulfate adsorbed

to Mn- or Fe(III)-oxides as shown in other studies (*25*). The increase in As could be due to direct reduction as well as reductive dissolution of sorbents (*26, 27*). Initial metal concentrations were enhanced in the autoclaved controls apparently due to a changed geochemistry. However, no concentration changes over time were observed.

The release of U during Fe(III)-reduction was surprising as Fe(III)-reducing bacteria (FeRB) are known to remove U(VI) from solution through precipitation of the insoluble U(IV) mineral phase uraninite [UO_2(s)] (*28, 3, 4*). Unfortunately, we were unable to differentiate the redox state of U in the liquid phase. Release of complexed U(VI) due to carbon amendments is unlikely due to the long lag phase observed prior to U mobilization. The formation of soluble Ca−uranyl−carbonate complexes that would be persistent to enzymatic reduction (*29, 30*) might be insignificant due to the lack of carbonates in the soil and the lack of CO_2 in the microcosm headspace at the beginning of incubation. Sequential extractions showed that ~30% of U is present in the amorphous Fe(III)-oxide fraction (*13*) and 81% of the solid phase-associated U was U(VI). These results indicate that U was sequestered in Btcl horizon that represents a redox transition boundary with likely active Fe remineralization processes. The incorporation of U into newly formed minerals was supported by the dip in U concentrations in the porewater in the Btlc horizon (Figure 1). Our results suggest that solid-phase associated U was not enzymatically reduced by FeRB but was released during reductive dissolution of Fe(III)-oxides. The partial decrease of U started with the onset of sulfate-reduction, although a contribution of FeRB cannot be ruled out. Several sulfate-reducing bacteria (SRB) reduce U enzymatically (*4*), although only a few have been reported to gain energy for growth from this process (*31*). In addition, sorption of U to metal sulfides, such as pyrite, might be possible (*32*). Comparative spectra demonstrated that no UO_2(s) was formed but apparently a sorbed complex of U(IV).

Metal immobilization during sulfate-reduction was probably due to formation of metal sulfides with Co, Ni, Zn, Cd, and As (*33−37*), although precipitation onto newly formed Fe(II) phases can not be excluded (*28, 38*). Since As concentrations were below those previously reported to facilitate arsenic sulfide precipitation (*35*), formation of As-bearing pyrite might have occurred (*39*).

The initial release of U during reoxidation is likely due to chemical oxidation by oxygen (*40*) followed by the sorption on or coprecipitation with newly formed Fe(III)- and Mn-oxides acting as metal scavengers (*41, 42*). Solubilization of Ni and Cd could be associated with the dissolution of metal sulfides (*43*). The oxidation of Fe(II) proceeded rather slowly.

Active Microbial Communities. In lactate-stimulated soil microcosms a diverse community dominated by *Acidobacteria* (*Geothrix*) and *Firmicutes* (*Pelosinus*) was detected. The genus *Geothrix* is known to reduce nitrate, Mn(IV), Fe(III), and humic acids (*44*), and although it is not known to reduce U, it might promote indirect reduction by formation of Fe(II) (*45*). *Geothrix* has been detected in biostimulated uranium-contaminated sediments, although its specific role in bioremediation is unclear (*45−47*). Members of the *Firmicutes* are able to transfer electrons from fermentation processes to Fe(III) (*48*). In addition, this group was suggested to play a role in bioremediation (*49*) due to observed reduction and increased sorption of U(VI) by *Firmicutes* (*50, 51*). A *δ-Proteobacteria* clone related to *P. propionicus*, and *β-Proteobacteria* clones related to *Dechloromonas* and *Janthinobacterium* were detected in the active FeRB community. Members of the genus *Pelobacter* are known to reduce Fe(III) and elemental sulfur, although it was suggested recently that Fe(III)-reduction only occurred indirectly via sulfide production (*52, 53*). The genus *Dechloromonas* has been observed before in uranium-contaminated soil microcosms (*49, 54*), and can reduce nitrate, sulfate, and apparently Cr(VI) and Se(VI) (*55, 56*). Dissimilatory Fe(III)-reduction has not been observed in pure cultures of *Dechloromonas* but by *Ferribacterium limneticum* which shared >96% identity to the *Dechloromonas*-related clones (*57*). Members of the genus *Janthinobacterium* have been reported to reduce nitrate (*58, 59*) and may be contributing to nitrate-, Fe(III)- and sulfate-reduction in the soil microcosms. Only a minor shift in the microbial community was detected between the sampling times. However, sulfate concentrations indicated the onset of sulfate-reduction starting at the time of sampling, whereas reduction of millimolar amounts of Fe(III) continued. Thus, the labeling of the FeRB community continued, while the labeling of the SRB community may have been below detection.

In agreement with former studies, addition of ethanol stimulated, in particular, the activity of *Geobacter*, a genus of the *δ-Proteobacteria* important in Fe(III)- and U(VI)-reducing communities (*60, 7, 8, 49, 54*). In ethanol microcosms, *Acidobacteria* (*Geothrix*) and *Firmicutes* (*Desulfosporosinus*) were detected in lower abundance after 14 days of incubation but became important members of the community after 34 days. *Desulfosporosinus* species are able to reduce sulfate and As(V) (*61*) and these organisms may have been involved in As solubilization at the beginning of the experiment. The increased TRF intensity of *Desulfosporosinus*-related clones during sulfate-reduction suggests that this organism contributed to this process. *Geothrix* can utilize acetate but not ethanol as electron donor, which explains its low abundance during ethanol consumption after 14 days of incubation (*44*). However, *Geothrix*, in addition to *Pelobacter*-related organisms who were detected after 34 days, might have contributed to Fe(III)-reduction during the later period of the incubation.

In contrast to biostimulation studies at uranium-contaminated sites in the United States (*7, 28, 51, 60*), uranium release was observed under reducing conditions in conjunction with activity of the *Geobactereaceae* family. Our results suggested that biostimulated FeRB facilitated the release of heavy metals, including U, and metal-enriched soil horizons could potentially be a contaminant source to groundwater and adjacent creek waters. Thus, no single bioremediation strategy, e.g., addition of identical carbon sources, can be applied to environments regardless if the same groups of FeRB are present.

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Supporting Information Available

Methods for XANES and fitted XANES spectra (Figure S1); identification of metabolically active bacteria; DNA concentrations in gradient fractions (Figure S2); similarity analysis of TRFLP patterns from SIP soil microcosms (Figure S3); TRFLP profiles from soil microcosms (Figure S4); and SSU rRNA gene cloning (Table S1). This information is available free of charge via the Internet at http://pubs.acs.org.

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Supporting Information

Impact of Biostimulated Redox Processes on Metal Dynamics in an Iron-rich Creek Soil of a former Uranium Mining Area

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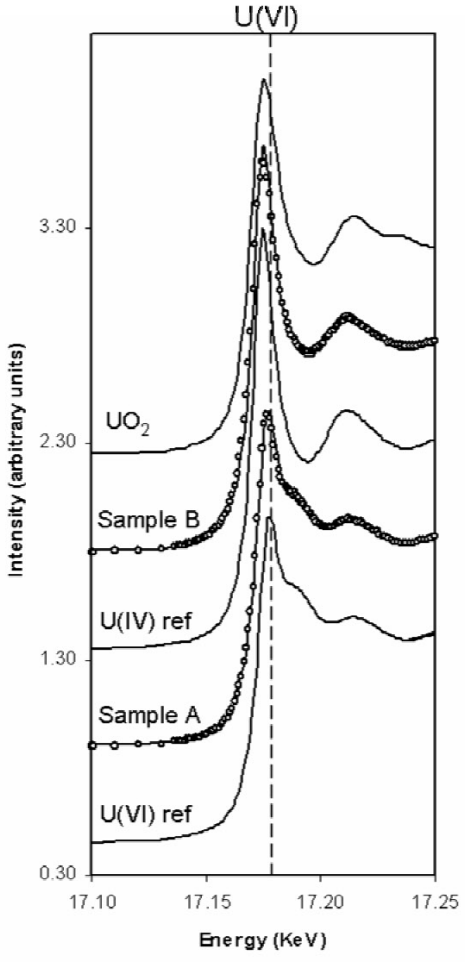
X-Ray Absorption Spectroscopy (XANES)

Uranium L_{III}-edge spectra of the samples were collected in fluorescence mode using a 13-element high purity germanium detector (Canberra) together with a digital signal processing unit (XIA) and a helium cryostat at 15K (*1*). Dead time correction of the fluorescence signal, energy calibration and the averaging of single scans were performed with the software package SixPack. Normalization, transformation from energy into k space, subtraction of a spline background as well as linear combination fits of the XANES region of the spectra using U(IV) and U(VI) reference spectra were performed with WinXAS using routine procedures (*2*).

FIGURE S1: Fitted XANES spectra of the GoII soil before (sample A) and after (sample B) anoxic incubation with reference spectra of U(IV), U(VI) used for the fitting as well as that of UO_2. In the spectra of samples A and B, the open circles represent data points and the solid line is the fit to the data obtained from linear combination fit using U(IV) and U(VI) components. The vertical dashed line indicate the U(VI) peak position which is clearly separated from the U(IV). A reference spectrum of U(IV) aqueous complex was chosen for the U(IV) component, while a spectrum of sorbed U(VI) on clay mineral was selected for U(VI).



Identification of Metabolically Active Bacteria

DNA Extraction and Separation of ^{13}C- and ^{12}C-labeled DNA. DNA was extracted from SIP samples with the PowerSoil DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA, USA) using the alternative lysis method. Triplicate DNA extracts were combined for each microcosm sample and ^{13}C- and ^{12}C-DNA were separated by density gradient centrifugation according to standard methods (*3*) with slight modifications. Gradients containing ethidium bromide were centrifuged in an Optima Max Ultracentrifuge (Beckman Coulter, Inc., Fullerton, California) at 65,000 rpm (176,267 × g_{avg}) in a TLA-110 rotor at 20°C for 36-40 hours. After centrifugation, DNA was retrieved by the gradient fractionation method, cleaned, and precipitated by a method described previously (*3*). DNA concentration was determined in each fraction spectrophotometrically and only the DNA containing fractions were used for further analyses.

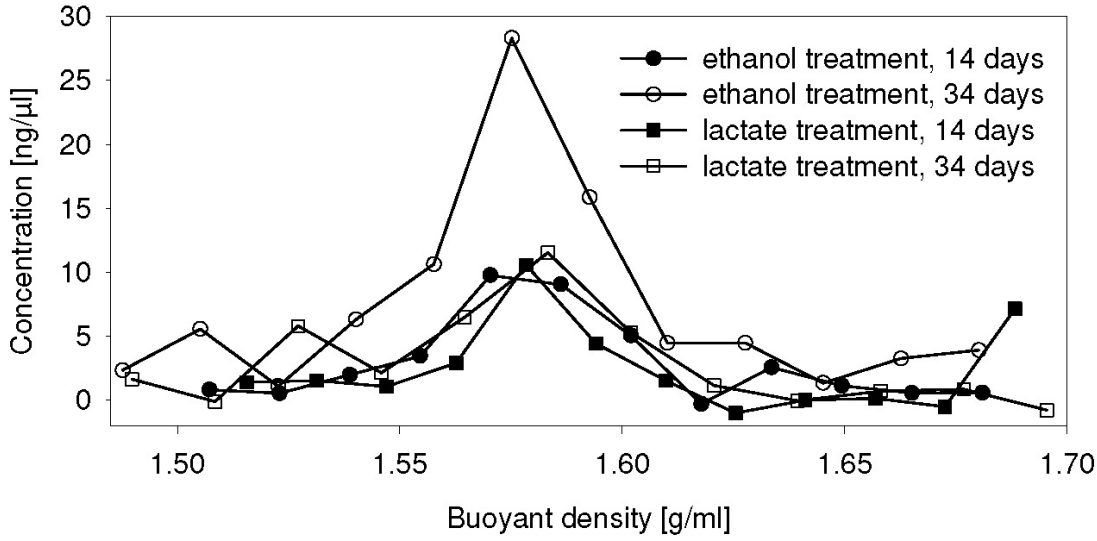
SSU rRNA Gene PCR Amplification and Terminal Restriction Fragment Length Polymorphism (TRFLP) Analysis. PCR amplification using the general domain *Bacteria* SSU rRNA gene primers 27F (5'-AGR GTT TGA TCM TGG CTC AG-3') (*4*) and 1492R (5'-GGT TAC CTT GTT ACG ACT T-3') (*5*) was performed as described previously (*6*). Fluorescently labelled PCR products for TRFLP were purified using the QIAquick PCR purification Kit according the manufacturer's instructions (QIAGEN Inc., Valencia, CA) and quantified as described above. For clone libraries from each carbon source equal volumes of PCR products were combined and purified by gel extraction using the QIAquick Gel Extraction Kit (QIAGEN Inc., Valencia, CA) according to the manufacturer's instructions.

Purified PCR products (100 ng) were digested with the restriction enzymes *Mnl*I and *Msp*I (New England Biolabs, Beverly, Massachusetts) and prepared for TRFLP as described previously (*6*). Samples were run on the ABI 3730 genetic analyzer (Applied Biosystems, Foster City, California) using GeneMapper software as described previously (*7*). Only TRFLP profiles

with a cumulative peak height ≥ 2,000 fluorescence units were used and peaks with a height < 50 fluorescent units were excluded from the analysis. All profiles were normalized to the total peak area. Pairwise similarities between profiles were calculated using Product-moment correlation distance matrix incorporating the presence/absence of TRF and clustered by UPGMA.

SSU rRNA Gene Clone Library Construction and Phylogenetic Analyses. Purified PCR products were combined from all density gradient fractions of each carbon treatment, and then cloned into the TOPO TA cloning vector pCR 2.1 according to manufacturer's instructions (Invitrogen, Carlsbad, CA). Cloned inserts were sequenced bidirectionally using vector specific primers (M13F/R) then assembled using BioEdit version 7.0.5.3 (*8*). Prior to phylogenetic analysis, vector and primer sequences flanking the gene inserts were removed. Clones were grouped into phylotypes based on 97% sequence similarity. Previously identified sequences with high sequence similarity to the clones obtained in this study were determined using the BLAST algorithm against the GenBank database available from NCBI (*9*). Clone sequences were checked for chimeras using the program Chimera Check from the Ribosomal Database Project II (*10*). All clone sequences were aligned with the alignment tool by Greengenes (*11*, accessible at http://greengenes.lbl.gov) and neighbor-joining trees incorporating a Jukes-Cantor distance correction were created from the alignments in the software *MEGA* version 4 (*12*). Statistical analyses were used to determine the sampling efficiencies, coverage and diversity differences within and between clone libraries (*13*; *14*). Rarefaction curves were calculated using Analytic Rarefaction 1.3 (*15*; *16*). Terminal restriction fragments (TRFs) of the clones were determined by in-silico cutting using the online program insilico.ehu.es (*17*). Sequences generated in this study were deposited in the GenBank database under the accession numbers GQ342318 to GQ342377.

1 **FIGURE S2:** DNA concentrations in the gradient fractions of different buoyant density.



2

FIGURE S3: Dendrograms representing the similarity of *Msp*I and *Mnl*I TRFLP patterns for the density gradient fractions obtained from SIP soil microcosms amended with ^{13}C-ethanol (A) or lactate (B) from both time points. Clustering analysis was based on the Sorensen's index, considering the presence/absence of peaks. The heavy fractions are indicated in bold letters.

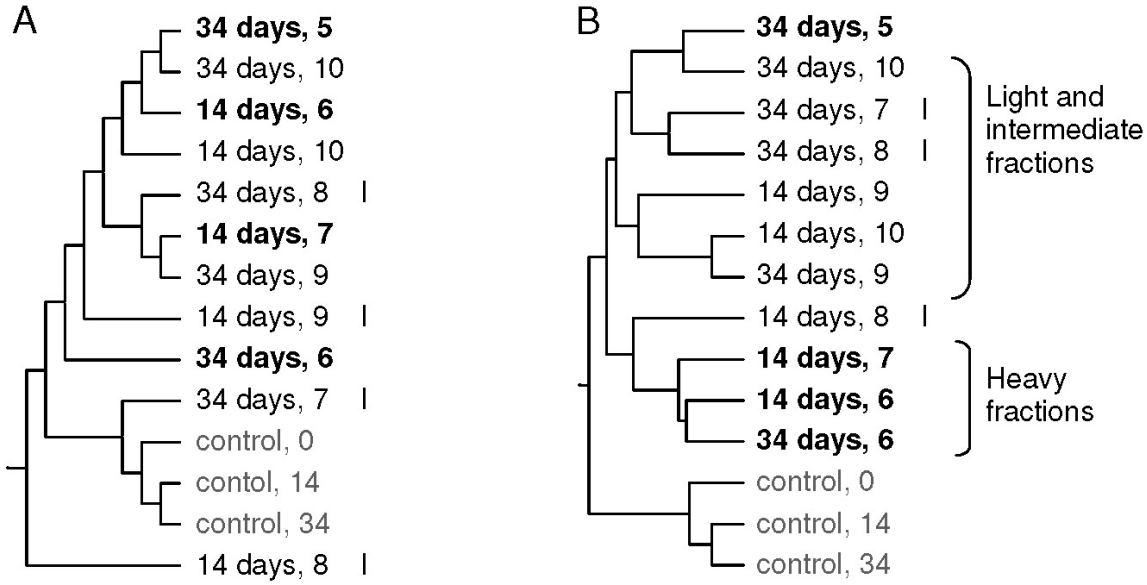


FIGURE S4: TRFLP profiles generated for ^{13}C-lactate, ^{13}C-ethanol, and unamended control soil microcosms on days 14 and 34. Terminal fragments were generated by digestion of SSU rRNA gene amplicons with the enzyme *Msp*I. Peaks identified by in silico digests are indicated.

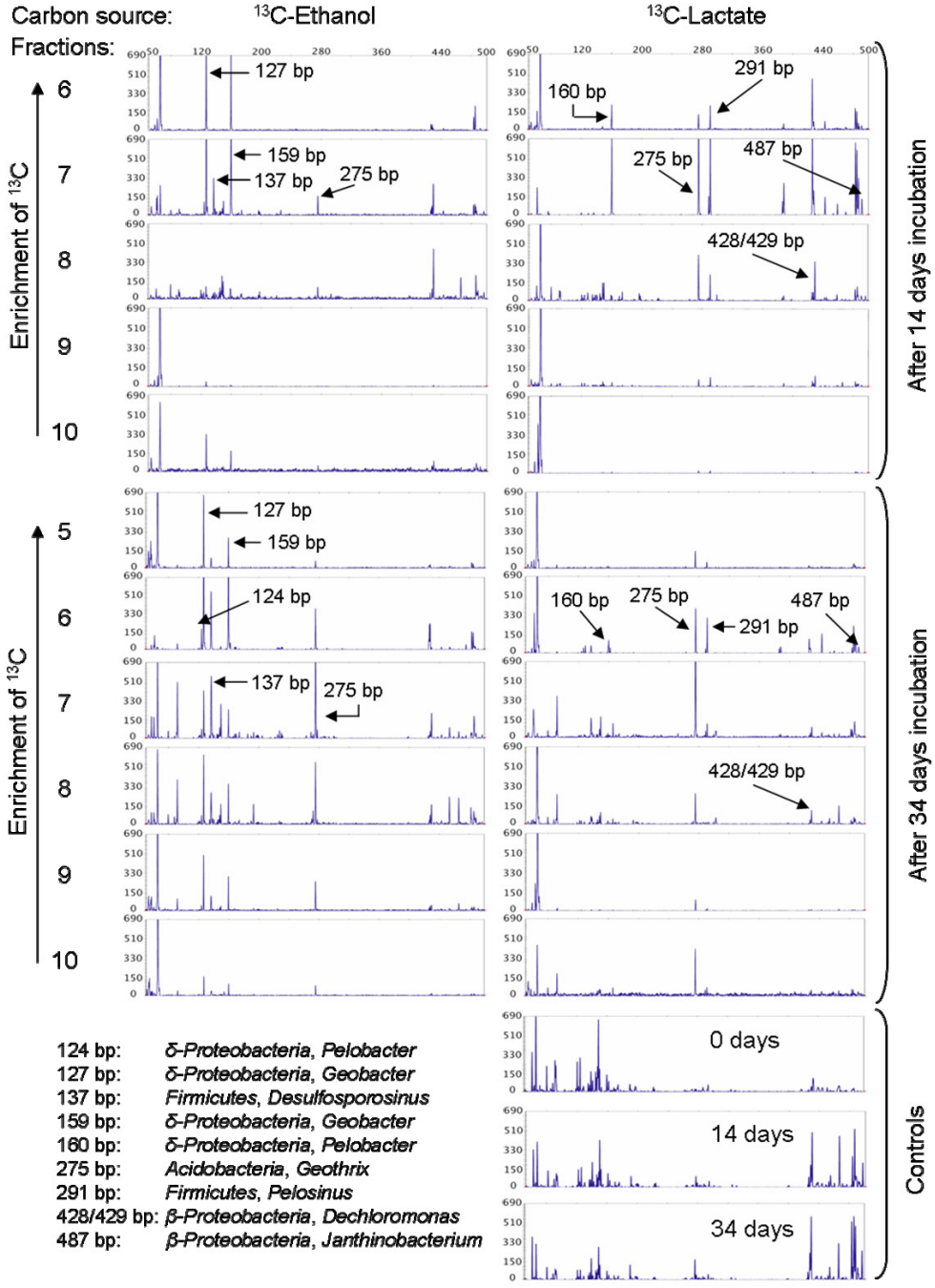


TABLE S1: Summary of phylogenetic affiliation and distribution of SSU rRNA gene clones from clone libraries of microcosm samples.

Phylogenetic group	Clone designation	Nearest relative (accession number)	% Identity	Number of clones			TRF[1] length [bp]
				Total	Ethanol treatment	Lactate treatment	
Acidobacteria	1OE3	Uncultured bacterium (AB240373)	98	1	1	0	152
	1OE5	Uncultured bacterium DA023 (Y07586)	98	1	1	0	201
	1OE20	Uncultured bacterium (AB240249)	97	2	1	1	280[3]
	2OE14	uncultured bacterium (AB240249)	98	8	1	7	278
	2OL2-5	Uncultured bacterium (AB240249)	98	1	0	1	278
	4OLI-9	Uncultured bacterium (AB240249)	98	1	0	1	278
	4OLI-11	Uncultured bacterium (AB240249)	97	1	0	1	278
	1OE40	clone FTLM5 (AF529125)	97	1	1	0	n.d.[2]
	2OE1	clone JH-WH251 (EF492907)	93	1	1	0	97
	2OL3-3	clone JH-WH251 (EF492907)	93	2	1	1	97
	3OE3	clone AH70 (AY963363)	99	2	2	0	435
	3OL11	clone FAC2 (DQ451441)	95	1	0	1	180
	4OLI-13	clone JH-WH272 (EF492908)	99	1	0	1	142
	4OLII-11	clone JH-WH272 (EF492908)	99	1	0	1	142
	4OLI-24	clone FAC8 (DQ451447)	96	1	0	1	95
	4OLII-12	clone C40 (EU234275)	95	1	0	1	138
	4OLII-16	clone RB41 (Z95722)	96	1	0	1	97
	4OLI-23	clone JH-WHS37 (EF492962)	98	1	0	1	152
α-Proteobacteria	1OE28	clone KSC2-39 (DQ532286)	99	1	1	0	152
	3OL8	clone FCPT726 (EF516954)	98	1	0	1	148
β-Proteobacteria	1OE2	*Polaromonas* sp. JS666 (CP000316)	98	1	1	0	492
	4OLI-5	soil clone 2_G8 (EU589321)	96	1	0	1	114

S10

	2OL2-30	clone 655087 (DQ404747)	98	1	0	1	491
	2OL48	clone Won90(0714) (DQ839505)	97	1	0	1	488
	4OLI-12	clone Won90(0714) (DQ839505)	98	1	0	1	488
	4OLII-6	clone TS29 (AB378592)	98	1	0	1	490
	1OE10	*Thauera* sp. R-28312 (AM084110)	99	1	1	0	432
	1OE17	*Dechloromonas* sp. MissR (AF170357)	98	2	1	1	428[3]
	3OL6	*Dechloromonas* sp. MissR (AF170357)	99	1	0	1	428
	3OE5	*Dechloromonas* sp. SIUL (AF170356)	97	1	1	0	430
	4OLI-6	*Dechloromonas aromatica* RCB (CP000089)	99	1	0	1	430
	2OE4N	clone ADK-MOh02-42 (EF520482)	99	1	1	0	433
	2OE8N	clone ADK-MOh02-42 (EF520482)	99	1	1	0	433
	2OL2-17	Uncultured bacterium (AB240462)	98	1	0	1	490
	3OE2	Uncultured bacterium (AB240462)	98	1	1	0	490
	5OL2N	clone SRRT09 (AB240462)	95	1	0	1	490
	5OL24N	clone VIR_B4 (EF565154)	92	1	0	1	446
γ-Proteobacteria	1OE22	clone EV818SWSAP22 (DQ337067)	96	2	2	0	490
	1OL1	Uncultured bacterium (AB255087)	95	1	0	1	n.d.[2]
	2OL7	clone B-AC40 (AY622251)	99	1	0	1	445
	4OLI-17	*Thiotrichaceae* clone D10_10 (EU266783)	98	1	0	1	140
δ-Proteobacteria	2OE11	*Geobacter* sp. M21 (EF527232)	99	14	14	0	130[3]
	3OE1	*Geobacter uraniireducens* Rf4 (CP000698)	96	1	1	0	161
	1OE15	clone pLW-45 (DQ067029)	98	2	2	0	128
	4OLII-20	clone: BS055 (AB240241)	97	1	0	1	161
Chlorobi	1OL9	Uncultured bacterium (AM086119)	95	1	0	1	453
Firmicutes	2OL5	*Pelosinus* sp. UFO1 (DQ295866)	99	1	0	1	290
	4OLI-25	*Pelosinus* sp. UFO1 (DQ295866)	99	1	0	1	292

	5OL22N	*Pelosinus* sp. UFO1 (DQ295866)	95	2	0	2	292
	2OL33	*Pelosinus* sp. UFO1 (DQ295866)	99	1	0	1	292
	4OLI-31	eubacterium from anoxic bulk soil (AJ229194)	97	1	0	1	522
	1OE44	*Desulfosporosinus* sp. clone CC_06 (EF565948)	96	1	1	0	139[3]
	4OLII-8	*Desulfosporosinus* sp. A10 (AJ582756)	97	1	0	1	140
	4OLII-21	clone 2E7_cons (EF688166)	96	1	0	1	280
Nitrospirae	2OE10	clone 35-52 (DQ833491)	97	1	1	0	366
	4OLI-21	clone Biofilm_94d_c2 (DQ058678)	89	1	0	1	682
	4OLI-22	clone CM138 (EF580980)	98	1	0	1	636
Planctomycetes	1OE33	clone FCPU701 (EF516278)	98	1	1	0	n.d.[2]
	4OLII-10	clone FFCH5701 (EU135162)	96	1	0	1	214
	4OLII-18	clone D15_04 (EU266844)	95	1	0	1	143

[1] TRF = terminal restriction fragment determined by in silico digest with *MspI*

[2] n.d. = not detectable

[3] confirmed by TRFLP profiling

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Microbial links between sulfate reduction and metal retention in uranium- and heavy metal-contaminated soil

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Abstract

Sulfate-reducing bacteria (SRB) can affect metal mobility either directly by reductive transformation of metal ions, e.g., uranium, into their insoluble forms or indirectly by formation of metal sulfides. This study evaluated *in situ* and biostimulated activity of SRB in groundwater influenced soils from a creek bank contaminated with heavy metals and radionuclides within the former uranium-mining district, Ronneburg (Germany). *In situ* activity of SRB, measured by the $^{35}SO_4^{2-}$-radiotracer method, was restricted to reduced soil horizons with rates ≤ 142 ± 20 nmol cm^{-3} day^{-1}. Concentrations of heavy metals were enriched in the solid phase of the reduced horizons, whereas porewater concentrations were low. XANES measurements demonstrated that ~80% of uranium was present as reduced uranium, but appeared to occur as a sorbed complex. Soil-based *dsrAB* clone libraries were dominated by sequences affiliated with members of the *Desulfobacterales,* but also *Desulfovibrionales*, S*yntrophobacteraceae* and *Clostridiales*. ^{13}C-acetate and ^{13}C-lactate biostimulated soil microcosms were dominated by sulfate and Fe(III) reduction. These processes were associated with enrichment of SRB and *Geobacteraceae*; enriched SRB were closely related to organisms detected in soils using the *dsrAB* marker. Concentrations of soluble nickel, cobalt, and occasionally zinc declined ≤ 100% during anoxic soil incubations. In contrast to other studies, soluble uranium increased in carbon-amended treatments reaching ≤ 1407 nM in solution. Our results suggest that (i) on-going sulfate reduction in contaminated soil resulted in *in situ* metal attenuation and (ii) the fate of uranium mobility is not predictable and may lead to downstream contamination of adjacent ecosystems.

Introduction

Dissimilatory sulfate-reducing bacteria (SRB) play an important role in the sulfur cycle and the mineralization of organic matter in anoxic marine and freshwater environments (54). In addition, sulfate reduction can occur in oxygenated habitats where anoxic niches (8) and the expression of superoxide reductase activity (34) provide protection for SRB against oxygen toxicity. The rate limiting step of sulfate reduction is catalyzed by the dissimilatory (bi-)sulfite reductase (coded by the *dsrAB* gene). Phylogenetic investigations have shown that this key enzyme for sulfate and sulfite respiration was present in early ancestors of modern *Bacteria* and *Archaea* (67).

Dissimilatory sulfate reduction has been shown to be a terminal-electron-accepting process (TEAP) in acid-mine drainage (AMD)-impacted and radionuclide- and metal-contaminated environments. Sulfate-reducing activities as well as SRB abundances show a wide range in these habitats (24, 29, 70). SRB are able to reductively transform metal ions, e.g., uranium and chromium, into insoluble and chemically inert forms via direct enzymatic reduction (41, 42). Sulfide, the end product of microbial sulfate reduction, may further contribute to metal attenuation through reduction of metal oxycations and oxyanions, such as those of uranium and chromium (4, 19), or through precipitation of metal cations as sulfides (15, 20). In addition, SRB have the potential to enhance metal retention via extracellular binding, cellular uptake and accumulation of metals, oxidation/reduction processes, surface mediated mineral precipitation (20, 53). Metal stress for SRB in uranium-contaminated sediments (48, 64) and biofilms from Pb-Zn deposits (39) can be reduced by the formation of uraninite and metal sulfides.

Previous work in uranium-contaminated environments has emphasized the role of biostimulated SRB on mediating uranium and/or technetium reduction (3, 46, 64), although other metal contaminants are present (56, 64). Long term stability of immobilized, reduced contaminants is a concern due to the potential for remobilization after stopping carbon addition. Therefore, it is important to understand alternative remediation processes, such as those involved in natural attenuation. In the former uranium-mining district Ronneburg (Thuringia, Germany), leaching of low-grade black shale by acid mine drainage and sulfuric acid and pyrite oxidation resulted in serious large scale contamination with heavy metals and radionuclides (28). Metal- and sulfate-enriched seepage waters and surface runoff infiltrated adjacent soils and surface waters leading to elevated concentrations of sulfate, nickel, copper, cadmium, zinc, arsenic and uranium in creek bank soils (9). At the Ronneburg site, the

presence of high levels of mixed contaminants provides a unique environment to look at complex processes involved in natural attenuation of contaminants. It is hypothesized that resident SRB contribute to natural uranium and heavy metal attenuation at the Ronneburg site. Thus, the objective of this study was to resolve the potential importance of SRB in contaminated creek bank soils both *in situ* and in biostimulated soil microcosms using stable isotope probing (SIP).

Materials and methods

Site description. The study site is located on the bank of Gessen Creek, one of the main drainage systems for the former leaching heaps within the former uranium- mining site near Ronneburg (Thuringia, Germany, location: E 4510121, N 5635807, Gauss/Krueger Postdam coordinate system). In the luvic gleysol, two iron-rich groundwater-influenced oxidized horizons (BElc, Btlc) and two groundwater-influenced reduced horizons (Br1, Br2) could be distinguished by color and texture below the humus top horizon (Ah) and a yellowish horizon (BEw/Ah) (6). The reduced horizons were grey (upper Br1, 82-103 cm in depth) and black (lower Br2, 102-110 cm in depth) in color, respectively. The solid phase of Br2 had a high total sulfur and carbon content (up to 2.0% and 3.4%, respectively) and showed a low redox potential of -30 mV (37). In contrast, Br1 had only a sulfur content of 0.4% and a redox potential of 60 mV.

Sampling procedure. Soil was aseptically sampled and stored in plastic bags in the dark at 4°C for transport and until further processing the following day. For determination of the acid volatile sulfur fraction the soil was frozen at -20°C. Soil samples for total extraction were stored at 4°C in the dark prior to analysis. Porewater samples for determination of pH, redox potential and measurements of nitrate, sulfate and soluble heavy metal concentrations were sampled with Rhizon suction samplers (Eijkelkamp, Giessbeek, the Netherlands) monthly from June to November 2007 from the soil profile at ~10 cm depth intervals. Redox potential of porewater was measured directly after sampling and soil waters were stored at 4°C overnight prior to subsequent analyses.

Determination of total soil metal concentrations. Soils from the Br1 and Br2 horizons were air dried, then finely ground to < 63 µm sieve size. The ground Br1 and Br2 soils were digested with concentrated hydrofluoric acid, nitric acid and perchloric acid at 150 to 170°C

in a pressure digestion system (DAS, PicoTrace, Bovenden, Germany). Single elements were measured in the resulting solutions using ICP-OES for Fe (Inductively coupled plasma-optical emission spectroscopy; Spectroflame P FAV05, Spectro Analytical Instruments, Kleve, Germany) and ICP-MS for As, Cd, Co, Cu, Ni, U and Zn (Inductively coupled plasma-mass spectrometry; X-SeriesII, ThermoFisher Scientific, Bremen).

X-ray absorption spectroscopy for analysis of solid uranium. X-ray Absorption Near-Edge Structure (XANES) spectra were collected for soils of Br1 and Br2 at the Rossendorf Beamline (Grenoble, France). Samples were prepared under an anoxic atmosphere. Uranium L_{III}-edge spectra were collected in fluorescence mode at 15 K using a closed cycle He cryostat. In order to determine the relative proportions of U(IV) and U(VI) in these samples, linear combination fits of the XANES region of the spectra using reference spectra of U(IV) and U(VI) were performed. A reference spectrum of U(IV) aqueous complex was chosen for the U(IV) component, whereas a spectrum of U(VI) sorbed on clay mineral was selected for U(VI). Relative proportions of U(VI) and U(IV) obtained from the fits were within ±1% of the reported values. Additional details are described in a previous study (6).

Determination of acid volatile sulfur (AVS). AVS was determined by suspending 10 g of soil in 50% ethanol under a nitrogen flow. AVS was liberated as hydrogen sulfide by cold acid distillation for 1 hour after addition of 8 ml of 30% (w/v) HCl. The released H_2S was collected in 2% (w/v) zinc acetate and measured spectrophotometrically according to the method described by Cline (12).

Sulfate reduction rates. Sulfate reduction rates were determined using the $^{35}SO_4^{2-}$ radiotracer technique (17). Rate determinations were conducted on all soil horizons in October 2007 and April 2008 in replicates of three and five, respectively. Soil (3 g) was transferred to sterile 7.5 ml serum bottles, sealed with butyl rubber stoppers and flushed with sterile argon, then diluted with 3 ml with sterile, anoxic water. Soil suspensions were amended with $H_2{}^{35}SO_4$ (Hartmann Analytics, Braunschweig, Germany) to a final activity of 100 kBq cm^{-3} soil and incubated for 2 hours at 15°C. Incubations were stopped by transferring the soil suspensions to plastic bottles containing 10 ml of 20% (w/v) zinc acetate. The samples were stored frozen at -20°C until further processing. The formation of sulfide was analyzed by the combined

chromium and acid distillation as described by Fossing and Jørgensen (17). Dry weight of soil was determined after drying at 105°C for 24 hours to constant weight.

Enumeration of sulfate-reducing bacteria. A three-tube most probable number (MPN) technique (14) using 10-fold serial dilutions was used to enumerate cultivable sulfate-reducing bacteria in soils from the reduced horizons. For selective growth a modified Postgate C medium (7) was used with a final sulfate concentration of 10 mM and pH 6.2. The medium was amended with sodium acetate or lactate (final concentrations 5 mM) as an electron donor. From both horizons, an additional MPN series was prepared to which heavy metals (0.3 µM $CdCl_2$, 38.6 µM $ZnCl_2$, 1.5 µM $CuCl_2$, 16.4 µM $NiCl_2$, 0.6 µM $CoCl_2$, 23.2 µM $AlCl_3$) were added reflecting the maximum porewater concentrations in the soil profile. The culture tubes were incubated at 16°C in the dark for five months. Positive growth was scored by a decrease in sulfate concentrations and MPN values with 95% confidence limits were calculated after de Man (13). When the ratio between MPN values was above 8.87, the abundance of organisms was considered significant (2).

Soil microcosms. Anoxic soil incubation experiments were performed (i) to study microbial activity under anoxic conditions (soil from horizons Br1 or Br2) and (ii) to investigate active sulfate-reducing bacteria using stable isotope probing (SIP, soil from Br2 only). Soils samples were collected in May and June 2007 from the Br2 and in February and October 2008 from the Br1 horizon, respectively. With exception of the second experiment using Br1 soil, microcosms were constructed by loading 20 g of soil (fresh weight) into 150 ml incubation bottles under a sterile argon atmosphere, and then sealed with rubber stoppers and secured with aluminum caps. A mineral solution (50 µM $NaNO_3$, 70 µM NH_4Cl and 10 mM $MgSO_4$ reflecting *in situ* soil water concentrations) was added in a ratio of 1:5 (wt soil /v) in a mineral solution. Seventy-five grams of soil (fresh weight) was prepared into 500 ml incubation bottles as described above for the second Br1 horizon experiment (expt II) with the sulfate concentrations again adjusted to match *in situ* conditions. The soil was diluted with mineral solution in a ratio of 1:4 (wt soil/v) containing 6 mM $MgSO_4$ only. Triplicate bottles for all experiments were amended to a final concentration of 5 mM glucose, 10 mM lactate, 10 mM acetate or 10 mM ethanol as electron donors. Glucose and ethanol were not supplied in the SIP or the second Br1 experiment. ^{13}C-labeled acetate or lactate (10 mM final concentration; >99 atom % ^{13}C; Cambridge Isotopes, USA) were used for the SIP microcosms. Reduced

carbon concentrations, 3 mM acetate or lactate, were used for the second set of Br1 microcosms. Three replicates without an added carbon source served as a control. The pH of the microcosms was adjusted to 6.3 and 6.1 for Br1 and Br2, respectively and the microcosms were then shaken for 1 h prior to the incubation in the dark at 16°C. Microcosms were regularly sampled over a time period of 31 to 37 days using anoxic, aseptic techniques to measure SO_4^{2-}, NO_3^-, Fe(II), and Mn (expect for the Br1 microbial activity experiment). Soluble metal concentrations, pH, and redox potential were determined at the beginning and end of the anoxic incubation.

Analytical techniques. Porewater redox potential, pH, Fe(II), total Fe, nitrate, ammonia, sulfate, metals and arsenic were measured as described elsewhere (6). In the microcosms, pH, and Eh were measured in the soil suspension immediately after sampling. Soil suspensions were centrifuged at 2,300 × g for 3 minutes and the supernatant was filtered through 0.2 µm nylon membrane for further analysis. Nitrate and sulfate were analyzed by ion chromatography. Soluble manganese, assumed as Mn(II), was determined by the formaldoxime method (31). Soluble metal and arsenic concentrations in microcosms were determined as described for porewater samples. Concentrations of aliphatic fatty acids, sugars and alcohols were analyzed using high-performance liquid chromatography (HPLC) according to Reiche et al. (55) Headspace methane concentrations were determined by gas chromatography (Hewlett Packard Co 5980 series II) as previously described (36). Gas pressures in the microcosms were measured with a TensioCheck TC 1066 needle manometer (Tensio-Technik, Geisenheim, Germany). Sulfate concentrations in MPN cultures were analyzed in the supernatant after centrifugation at 8400 × g for 2 minutes using the barium chloride method (65).

Analysis of *dsrAB* functional genes. *DNA extraction and PCR amplification.* Soil samples (Br2) for DNA extraction were collected from the Gessen Creek bank in August 2007 and stored at -20°C. Genomic DNA was isolated and purified using the Power Soil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, USA) according to the manufacturer's instructions. Amplification of the dissimilatory sulfite reductase (*dsrAB*) gene was performed using a forward and reverse DSR primer mix targeting a variety of sulfate-reducing bacteria (52, 66). The PCR reaction contained 1x MasterAmp PCR Premix D (Epicentre Biotechnologies, Madison, Wisconsin USA), 2.5 U Taq polymerase (Jena Bioscience, Jena, Germany), DSR

primer mix with equimolar concentration of 0.1 μM for each primer variant and 3.5 ng soil DNA. Amplification was performed in three replicates under the following conditions: initial denaturation step for 3 min at 94°C, followed by 30 cycles of denaturation at 94°C for 40 s, annealing at 48°C for 40 s, elongation at 72°C for 90 s and a final elongation step for 10 min at 72°C (66). The ~1.9-kb *dsrAB* amplicon was purified with gel extraction using the Agarose Gel Extraction Kit (Jena Bioscience, Jena, Germany) according to the manufacturer's protocol. Purified *dsrAB* amplicons were cloned into the TOPO TA cloning vector pCR®4 according to the manufacturer's instructions (Invitrogen, Carlsbad, CA USA). Plasmids of clones containing the *dsrAB* gene were extracted with the Plasmid Mini-Prep Kit (Jena Bioscience, Jena, Germany) according to the manufacturer's instructions. *dsrAB* fragments were sequenced by Macrogen (Seoul, South Korea).

dsrAB phylogenetic analysis. Phylogenetic analyses were performed using the ARB program (44), where all nucleotide sequences (forward reads) were imported into a *dsrAB*/DsrAB-ARB database for SRB maintained at the Department of Microbial Ecology, University of Vienna (43) and aligned manually based on deduced amino acids. *DsrAB* clones with nucleotide sequence identity equal or greater than 90% with each other were grouped into an operational taxonomic unit (OTU). A consensus tree was based on Neighbor-Joining, maximum-parsimony (Phylip PROTPARS; 1000 bootstraps) and maximum-likelihood methods using a DsrA filter without correction for partial *dsrA* sequences (200 amino-acid positions) in the ARB program. Percentage coverage was calculated as the ratio between observed and expected OTU according to rarefaction analysis using Analytic Rarefaction 1.3 (22). Sequences of this study were published at the GenBank database under the accession numbers GU233963 - GU234006.

Stable isotope probing of ^{13}C- and ^{12}C-labeled small subunit (SSU) rRNA genes. Microcosm samples for stable isotope probing (SIP) were centrifuged for 3 minutes at 2,300 × *g*, then the pellets were stored frozen at -20°C until nucleic acid extraction. Procedures used for DNA extraction, separation of ^{13}C- and ^{12}C -labeled DNA, SSU rRNA gene PCR amplification and terminal restriction fragment length polymorphism (TRFLP) analysis were performed according to Burkhardt et al. (6) with slight modifications. In brief, triplicate DNA extracts from solid phase microcosm samples were combined, and then ^{13}C- and ^{12}C-DNA were separated by density gradient centrifugation. After centrifugation the DNA was retrieved by the gradient fractionation method, cleaned and precipitated. SSU rRNA genes were

amplified using the general domain *Bacteria* SSU rRNA gene primers 27F and 1492R, where for TRFLP analysis, the 27F primer was 5' end labeled with 6-carboxyfluorescein. For TRFLP, digested und purified DNA samples were run on the ABI 3730 genetic analyzer using GeneMapper software. The pairwise similarities between profiles were calculated from a matrix of the presence/absence of TRF using product-moment correlation distance matrix. Cluster analysis based on this similarity matrix was performed by UPGMA.

For SSU rRNA gene clone library construction and phylogenetic analyses**,** purified PCR products were ligated into the TOPO TA cloning vector pCR XL according to manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Ligations were shipped to the Genome Sequencing Center at Washington University (St. Louis, MO, USA) for transformation and bidirectional sequencing with vector specific primers (M13F/R). Sequences were assembled and vector sequences flanking the SSU rRNA gene inserts were removed using Geneious Pro version 4.6.4 (Biomatters, http://www.geneious.com). Clones were grouped into phylotypes based on a sequence similarity cut off of 97% and previously identified sequences with high sequence similarity to the clones obtained in this study were determined using the BLAST algorithm against the GenBank database available from National Center for Biotechnology Information (NCBI). All clone sequences were aligned with the alignment tool by Greengenes and nearest neighbors were identified using the Classify tool against the Greengenes database. Sequences generated in this study were deposited in the GenBank database under the accession numbers GU235998 to GU236099.

Results

Soil geochemistry. The average pH of the soil water measured monthly was neutral to slightly acidic, ranging from 5.8 to 7.0 over the whole profile (Fig. 1A). In general, the redox potential was low in both reduced horizons. Sulfate was enriched in the porewater (Fig. 1A) with average concentrations of 3.4 ± 3.2 mM and 3.1 ± 2.3 mM for Br1 and Br2, respectively. However, the range of sulfate concentrations for the reduced horizons was from 0.3 mM to 12.4 mM over the sampling period. Nitrate (Fig. 1A) was negligibly low in the reduced horizons (< 20 µM), whereas it reached up to 1.2 mM in the upper horizons during the sampling period (data not shown). Both manganese and Fe(II) increased with depth, reaching highest concentrations in the horizon Br2 at 0.1 mM Mn and 2.2 mM Fe(II), while Fe(III) was negligibly and declined within the oxidized horizons (Fig. 1B). Soluble heavy metal concentrations (U, Co, Ni, Zn and Cu) peaked in the oxidized Btlc horizon but were low in

the deeper reduced horizons (Fig. 1C). The maximum uranium concentration of 278 nM was observed in horizon Br2 in June 2007. In general, arsenic concentrations were higher in the reduced horizons reaching up to 180 nM compared to the upper, oxidized horizons (Fig. 1C).

Acid volatile sulfur (AVS) was higher in the solid phase of the reduced horizons (52 and 126 mmol kg [dry wt soil]$^{-1}$ at Br1 and Br2, respectively), whereas much lower concentrations were observed in the upper soil horizons (AVS $\leq$ 0.2 mmol kg [dry wt soil]$^{-1}$). Creek bank soil contained high content of metals in the solid phase and exceeded background levels in the majority of cases (Table1). Br2 soil accumulated total uranium, zinc, nickel, and copper to a higher extent than Br1 soil (Table 1). According to XANES measurement, soil of both horizons was highly enriched in reduced uranium species, with Br2 (83.8%) containing a slightly higher amount of U(IV) than Br1 (79.5%). In addition, a comparison of samples Br1 and Br2 to a uraninite (UO_2) reference spectrum showed a lack of features in the post-edge region of the two samples, suggesting that U(IV) in these samples did not occur in a crystalline form similar to UO_2, but was more likely to be present as a sorbed complex (Fig. 2).

Sulfate reduction rates (SRR). Sulfate-reducing activity was restricted to the reduced horizons (Fig. 1D) with sulfate reduction rates of 142 ± 20 nmol cm^{-3} day^{-1} and 48 ± 41 nmol cm^{-3} day^{-1} for Br1 and Br2 in October 2007, respectively. Sulfate reduction rates determined in April 2008 showed a similar pattern, but total rates were only 5 nmol cm^{-3} day^{-1}. In contrast, activity was below detection in the upper, oxidized horizons.

Enumeration of sulfate-reducing bacteria. The abundance of cultivated SRB (Table 2) was similar in horizons Br1 and Br2 but differed slightly according to the amended electron donor in the enrichments. Highest abundances were observed in lactate-amended cultures of the Br2 horizon. SRB abundance in both horizons was slightly lower in the presence of heavy metals (Table 2). The difference was only significant in the lactate treatment for Br2.

***dsrAB* soil community.** Partial *dsrAB* sequences were analyzed to identify soil-associated SRB. A clone library was constructed with 109 clones screened resulting in a coverage of 76%. Sequences grouped into 39 OTUs and all were closely related to uncultured SRB from pristine environments as well as a uranium-mining site, a leachate-polluted aquifer and sediment from a polluted harbor. *dsrAB* clones grouped within the families *Desulfobacterales*

(64% of total clones), *Desulfovibrionales* (2% of total clones) and *Syntrophobacterales* (17% of total clones) within the *Deltaproteobacteria* and the *Clostridiales* (15% of total clones) within the *Firmicutes* (Fig. 3). The phylogenetic affiliation of few clones (3% of total clones) remained uncertain (Fig. 3). Using *dsrAB* primers, the non-SRB, *Carboxydothermus hydrogenoformans*, which is known for sulfite reduction (23), was also detected.

Reductive redox processes in anoxic soil microcosms. Microbial activity was stimulated via carbon amendment in four experiments. In all of the microcosms, the pH increased over the incubation period and was slightly higher in biostimulated treatments (pH 7.3) compared to the unamended controls (pH 6.6). The redox potential (Eh) declined in the biostimulated microcosms down to -305 mV, whereas changes in Eh were negligible for the controls (data not shown).

Nitrate was rapidly reduced in all treatments within the first two to four days with negligible nitrate reduction rates (Fig. 4) due to low *in situ* concentrations. Manganese was reduced in µM-scale range within the first ten to sixteen days in Br1 soil and showed a slight decrease after a plateau phase (Fig. 4). In contrast, manganese concentrations of Br2 microcosms did not change significantly (data not shown). Formation rates of Fe(II) were not consistent throughout the different experiments, but some Fe(III) was reduced within the first five to 16 days for both soil horizons in two of four non-biostimulated and in five of 12 biostimulated treatments if the initial Fe(II) soil content was low (Fig. 4 and data not shown). Net sulfate reduction was the dominant TEAP and trends were similar in all experiments (Fig. 4 and data not shown). Net sulfate reduction began only after a 12 or 17 day lag-phase, after slight Fe(III) reduction had ceased (Fig. 4). Due to an accumulation of sulfate in the Br2 control microcosms, a slight decreasing trend in sulfate was observed in the carbon-amended treatments after five days (Fig. 5A and B) but depletion was below detection. Kinetics of sulfate reduction were similar for Br1 and Br2 soil and highest stimulation was observed with the addition of acetate (rates up to 30.4 µmol g [fresh wt soil]$^{-1}$ day^{-1}) followed by lactate and ethanol. Methane was formed simultaneously as sulfate concentrations decreased in soil microcosms after 16 to 20 days with rates $\leq$ 0.3 µmol g (fresh wt soil)$^{-1}$ day^{-1} (Fig. 4) in acetate or lactate amended treatments.

Characterization of active microbial communities in anoxic soil microcosms. Addition of supplemental acetate and lactate stimulated SRB best and consumption of these substrates

was similar in all microcosm experiments (Fig. 5C, D for the SIP experiment). ^{13}C-acetate was consumed slowly during the incubation and a near-linear decrease was observed from days 18 to 34 (Fig. 5C). Most of the acetate was consumed concomitantly with sulfate reduction and all acetate was consumed by day 34 (Fig. 5A, C). In the ^{13}C-lactate-amended microcosms, lactate was consumed by day 6 of the incubation, yielding acetate and propionate as end products (Fig. 5D). Accumulated acetate was consumed slowly and appeared to be in parallel with sulfate reduction (Fig. 5B, D).

DNA was detected in gradient fractions with buoyant densities ranging from 1.52 to 1.62 g ml^{-1} (data not shown). The highest density DNA fractions represented the microbial community with the highest ^{13}C incorporation. Comparison of TRFLP patterns generated for each of the DNA fractions revealed a distinct clustering of the heavy fractions from days 4 and 34 of the ^{13}C-lactate treatment (data not shown). The majority of heavy fractions clustered together for the ^{13}C-acetate treatment, however we observed no distinct clustering as a function of incubation time. Cloning and sequencing was performed for the heavy fractions of each treatment for each time point. A total of 4 clone libraries were constructed and 62 and 50 clones were screened for the ^{13}C-acetate day 24 and 34 libraries, respectively, and 56 clones were screened for the ^{13}C-lactate libraries. Rarefaction analysis revealed that the diversity was not exhausted (data not shown). Phylotypes related to members of the *Deltaproteobacteria, Chloroflexi, Spirochaetes* and Candidate Divisions OP8, OP10 and OP11 were detected in all treatments and at all time points. In addition, members of the *Actinobacteria, Bacteroidetes, Firmicutes, Acidobacteria, Fusobacter, Nitrospirae, Planctomycetes* and *Verrucomicrobia* were detected. Members of the *Deltaproteobacteria* dominated all clone libraries and represented ~77% of all clones in the acetate treatment (Fig. 6). In the lactate treatment, the abundance of *Deltaproteobacteria*-related phylotypes increased from 44 to 70% of the total clones from day 4 to day 34. The majority of *Deltaproteobacteria* clones detected in all treatments were related to Fe(III)-reducing bacteria within the genus *Geobacter* (> 80% of *Deltaproteobacteria* clones; data not shown). Additional *Deltaproteobacteria* clones were related to members of the sulfate-reducing genera *Desulfobacca* and *Desulfocapsa*. The increase in *Deltaproteobacteria* at day 34 in the lactate treatment was due to the appearance of phylotypes related to SRB within the *Desulfobacteriaceae, Desulfovibrionaceae*, and *Desulfobulbaceae* families that are known to utilize acetate (10). Phylotypes related to the *Chloroflexi* phylum were equally abundant at both time points, representing 8 and 9% of acetate and lactate clone libraries, respectively

(Fig. 6). Members of the *Firmicutes* were only detected at day 24 in the acetate treatment but at both timepoints in the lactate treatment (Fig. 6). *Firmicutes* were in higher abundance at day 4 in the lactate treatment (14% of total clones) than day 34 (5% of total clones). The majority of *Firmicutes* phylotypes were related to *Pelosinus fermentans* that is known to ferment lactate and reduce Fe(III), and to *Desulfotalea psychrophila*, which reduces sulfate and utilizes lactate as a carbon substrate.

Effect of anoxic incubation on dissolved metal concentrations. Surprisingly, uranium was released to solution at the end of incubation in all carbon-amended treatments (Table 3 and data not shown). Concentrations of U reached up to 1407 nM in the Br2 soil suspension amended with acetate (235-fold increase), and 165 nM in the Br1 soil suspension amended with lactate (6.6-fold increase). Nickel and cobalt were immobilized in all treatments, with up to 100% of the soluble metals removed from solution (Table 3 and data not shown). The addition of carbon led to a 3.0-fold higher reduction in nickel concentrations at the end of the incubation. Similarly, cobalt concentrations were reduced to a 2.1-fold higher extent compared to the unamended controls. The dynamics of soluble zinc were not consistent among the treatments and experiments. A decline in soluble zinc, up to 95% of the starting concentration, was observed in soil microcosms for the Br1 and Br2 horizons (Table 3 and data not shown). whereas the soluble zinc concentrations in the second Br1 experiment declined in a similar manner for both the control and carbon amended treatments (Table 3). The low soluble copper concentrations were constant or even declined in Br1 soil microcosms (Table 3 and data not shown). While little to no change in soluble arsenic concentrations were observed in the second Br1 experiment, arsenic concentrations were up to 5.7-fold higher at the end of incubation in the other microcosm experiments (Table 3).

Discussion

On-going anaerobic microbial activities in Gessen Creek bank soil. Porewater profiles suggested that reduction of nitrate occurred mainly in the upper oxidized horizons, whereas soluble manganese and Fe(II) increased primarily in the reduced soil horizons Br1 and Br2. These horizons contained the highest concentrations of sulfate, which were highly variable with time. The accumulation of acid volatile sulfur and high *in situ* sulfate reduction rates in Br1 and Br2 soils indicated on-going dissimilatory sulfate reduction in the creek bank soil. The *in situ* sulfate-reducing activity was slightly higher than what was previously found in

contaminated soils and sediments of the Norilsk mining area (29) and was in the range of rates reported from unpolluted freshwater (25, 71) or marine (32, 58) ecosystems. A long, heavy rainfall prior to our measurements diluted sulfate concentrations, oxygenated the soil, and was likely responsible for the lower reduction rates observed in April 2008.

The detection of a diverse sulfate-reducing community via analysis of the functional marker gene *dsrAB* in the Br2 soil horizon supported our findings of on-going sulfate reduction. The majority of clones were related to the *Desulfobacterales*, and the closest relatives of our clones were freshwater-associated SRB that were found in uranium-mining tailings (11), metal-contaminated aquifers (21) or intertidal river soil (47). SRB abundance was within the same range as in samples from other uranium-contaminated subsurface sediments (48), deciduous forest soils (60) or lake sediments (35, 38). Abundances of resident SRB were only slightly inhibited in the presence of metals at maximum *in situ* porewater concentration. This indicates that the resident, cultivatable SRB community is adapted to the level of metal stress in the Gessen Creek bank soils.

Microbial activities during anoxic soil incubation. Sulfate reduction was an important TEAP in biostimulated, anoxic microcosms after 12 to 17 days of incubation; with amendment by acetate and lactate resulting in the highest sulfate reduction rates. *In situ* SRR were in the nmol-range, suggesting that sulfate reducers were active at a low-level also during the first days of incubation but masked due to the high sulfate concentrations in the treatments. The increase in sulfate concentrations that was observed in the Br2 control microcosms at the beginning of incubation may been caused by the desorption of sulfate bound or adsorbed to Fe(III)-(hydr)oxides (57). Because nitrate and manganese were present at low concentrations, nitrate and manganese reduction were likely not substantial energy-generating processes. Microbial Fe(III)-reduction was a significant TEAP next to sulfate reduction, although not steadily since Fe(II) formation rates were low and highly variable among treatments and replicates. However, Fe(II) may also have been formed from abiotic reduction coupled to sulfide oxidation, since a high reduced sulfur content was present in the in Br2 soil. Although we did not detect significant higher Fe(II) forming activities compared to the unamended control (data not shown), *Geobacter* was active in the lactate- and acetate-amended treatments. In Btlc soil, stimulated Fe(III) reduction yielded the presence of the genera *Geobacter, Geothrix* and *Pelosinus* (6). This observation was similar to previous field and laboratory

experiments at other uranium-contaminated sites, where Fe(III)- and metal-reducing bacteria were of great importance for bioremediation (26, 27).

Active biostimulated sulfate-reducing bacteria were shown to be related to SRB known for complete or incomplete acetate utilization (10) and the capability of oxidizing propionate which was observed in ^{13}C-lactate treatments (69). *Desulfobacca acetoxidans* and *Desulfocapsa thiozymogenes* related clones have been previously isolated from uranium and Pb/Zn mining sites (11, 63), indicating the presence of two metal- and radionuclide-tolerant species. The high abundance of *Geobacter* spp. found in the active communities may also have been involved in reduction of humic substances and elemental sulfur (40, 62), since soil contained high carbon and sulfur contents.

Metal retention potential. An accumulation of metals in the solid phase was not associated with high soluble concentrations of metals indicating an additional capacity for metal attenuation from the inherent soil-associated microorganisms and/or different soil properties. A decrease in soluble nickel and cobalt was observed independent of biostimulation, as expected based on previous work on sulfate reduction in batch experiments (7, 33). It is hypothesized that the decrease in nickel and cobalt was related to the formation of metal-sulfides in the soil incubation experiments. Therefore, on-going, *in situ* sulfate-reducing activity would be sufficient to precipitate nickel and cobalt as sulfides (16, 33). But passive mechanisms, such as metal-binding to sites on bacterial cell surfaces and to metal-complexing groups of extra-polymeric substances (20), cannot be ruled out. Metal dynamics for zinc, copper and arsenic were not uniform throughout the anoxic incubations of the soil, and were therefore in contrast to previous work that showed metal sulfide formation with these cations and anions under sulfate-reducing conditions (39, 50, 61). As(VI)-reducing bacteria, such as *Desulfosporosinus auripigmenti*, likely contributed to the observed increase in soluble arsenic (51). Therefore, sulfate-reducing bacteria have the potential to attenuate metal cations in the investigated field site, which was indicated by the metal geochemistry.

In contrast to what other studies have shown (e.g., 1, 72), we observed that soluble uranium concentrations increased under anoxic, sulfate-reducing conditions. This was unexpected because anaerobic metabolism of bacteria has been shown to promote reduction in soluble U by indirect and direct mechanisms (5, 42, 45). However, as was seen in this study, an increase in uranium concentration was previously observed during sulfate reduction in uranium-contaminated aquifers (3) or in pure cultures of *Desulfovibrio desulfuricans* G20

(59). It is also possible that bacteria from the soil produced siderophores, thereby promoting the dissolution of UO_2 (18) or that microbially mediated formation of carbonates could have resulted in highly stable carbonate-U(VI) complexes (68). However, carbonate-U(VI) complexes are unlikely to have been formed due to a lack of carbonates in the soil and also XANES data did not indicate the presence of carbonate-U(VI) complexes in the soil. Surface bound uranyl ions [U(IV)], ~20% of the uranium in the soil solid phase, may have been detached from Fe(III)-mineral phases which have a affinity for uranium (49) and which were bacterially reduced up on amendment of the microcosms. For example, the Fe(III)-mineral phase illite was likely to be present in Br1 and Br2 and could provide sorption surfaces for hexavalent actinides (30).

Conclusions. Active SRB were identified in reduced soils within the bank of Gessen Creek in the former uranium-mining site Ronneburg. SRB were shown to be adapted to the presence of metals and radionuclides and to influence the retention of contaminants. Especially nickel and cobalt, but occasionally zinc and copper, were retained during sulfate reduction indicating that soil-associated SRB contributed to *in situ* metal attenuation, which could explain the high solid metal contents and the low concentrations of metals in the porewater. Uranium was released during anaerobic microbial activities acting as potential source of contaminants to downstream ecosystems. The increase in soluble uranium concentrations is in contrast to what has been seen during biostimulation of iron- and sulfate-reducing bacteria at other uranium-contaminated sites (3, 27). Our results show that site-specific geochemistry and variable *in situ* microbial communities can affect the success of biostimulation as strategy for the enhancement of metal retention. In reduced soils of the Gessen Creek bank, on-going sulfate reduction is providing a means for natural attenuation of nickel and cobalt contaminants, but does not lessen the risk of downstream uranium contamination.

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Figure legends

Figure 1: Geochemistry (A-C) of the soil profile in June of 2007 and depth profile of sulfate reduction rates (SRR) (D) at the bank of the contaminated Gessen Creek. SRR values are averages ± standard deviations (October 2007 n = 3, April 2008 n = 5). The corresponding soil horizons are given at the right side of the graphs.

Figure 2: XANES: Fitted XANES spectra of the Br1 and Br2 samples with reference spectra of U(IV), U(VI) used for the fitting as well as that of UO_2. In the spectra of samples Br1 and Br2, the open circles represent data points and the solid line is the fit to the data obtained from linear combination fit using U(IV) and U(VI) components. The vertical dashed line indicate the U(VI) peak position which is clearly separated from U(IV).

Figure 3: Phylogenetic tree indicating the relationship of deduced dsrAB amino acid sequences retrieved from Gessen Creek bank soils (indicated by bold-face type) to those of cultivated sulfate-reducing bacteria and other environmental clone sequences. The tree represents a consensus of the phylogeny determined using neighbor-joining, maximum-parsimony, and maximum-likelihood methods on an alignment of 245 amino acids of the *dsrA* gene. Parsimony bootstrap values (1,000 data resamplings) ≥ 90% are indicated by closed circles and values ≥ 70% are indicated by open circles. The scale bar indicates the estimated number of amino acid changes per amino acid sequence position.

Figure 4: Electron-accepting processes represented by the Br1 microcosms amended with acetate. Values are means of triplicates ± standard deviations.

Figure 5: Electron acceptor and donor utilization in the SIP microcosms, amended with ^{13}C-acetate (A, C) and ^{13}C-lactate (B, D). Open boxes indicate sampling points for SIP. Values are means of triplicates ± standard deviations.

Figure 6: Frequencies of active bacterial phylogenetic lineages detected in SSU rRNA gene clone libraries from (A) ^{13}C-acetate- and (B) ^{13}C-lactate-amended microcosms. Calculations were made based on the total number of clones associated with phylotypes of sequenced representatives.

Figure 1

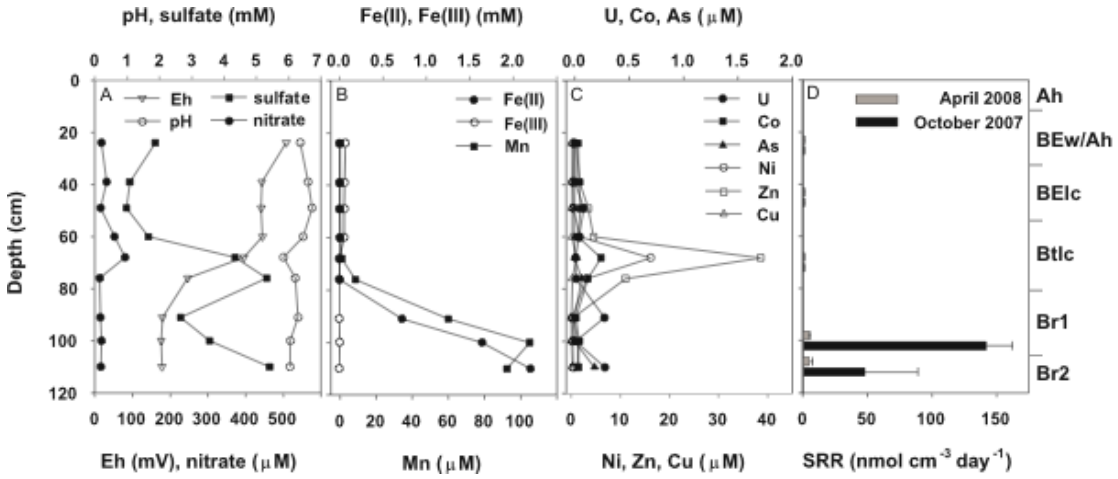
pH, sulfate (mM)
Fe(II), Fe(III) (mM)
U, Co, As (μM)
Depth (cm)
A
Eh
pH
sulfate
nitrate
B
Fe(II)
Fe(III)
Mn
C
U
Co
As
Ni
Zn
Cu
D
April 2008
October 2007
Ah
BEw/Ah
BElc
Btlc
Br1
Br2
Eh (mV), nitrate (μM)
Mn (μM)
Ni, Zn, Cu (μM)
SRR (nmol cm-3 day-1)

Figure 2

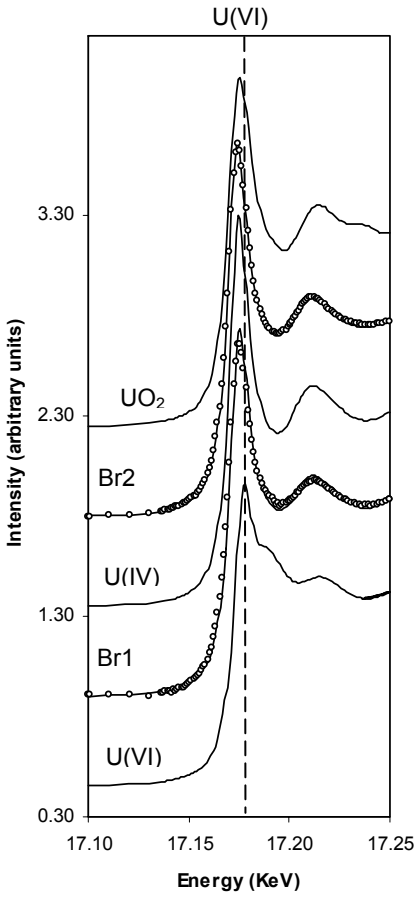
U(VI)
3.30
2.30
1.30
0.30
Intensity (arbitrary units)
UO2
Br2
U(IV)
Br1
U(VI)
17.10
17.15
17.20
17.25
Energy (KeV)

Figure 3


leachate-polluted aquifer clone LGWG16, ABK90663
I92_7 (2 clones)
leachate-polluted aquifer clone LGWI08, ABK90715
I57_20 (2 clones)
II18_31 (2 clones)
II_71
leachate-polluted aquifer clone, ABK90791
leachate-polluted aquifer clone LGWG24, ABK90681
II_69
freshwater clone B34, BAF42728
I86_23 (2 clones)
clone mXyS1, AAQ05941
marine sediment clone 25H-0D-5, ACJ11257
I40_1 (14 clones)
II14_27 (11 clones)
Desulfobacterium anilini, AF482455
Desulfotomaculum acetoxidans, AF271768
Desulfotomaculum geothermicum, AF273029
Desulfotomaculum kuznetsovii, AJ310431
Desulfotomaculum kuznetsovii, AF273031
Desulfotomaculum thermocisternum, AF074396
Desulfotomaculum thermobenzoicum, AJ310432
Desulfotomaculum thermoacetoxidans, AF271770
III_34
III_59
III_19
I17_6 (3 clones)
III_1 (2 clones)
marine sediment clone 40H-0D-32, ACJ11359
marine sediment clone 13CDSR2-33, CAJ41483
leachate-polluted aquifer clone LGWG06, ABK90673
II3_24
Desulfosalina propionicus, DQ386237
uranium-mining site clone P4D-17, AAV98379
II9_26
Desulfobacterium oleovorans, AF418201
Desulfatibacillum aliphaticivorans, AY504426
III_14 (3 clones)
acidic fen clone dsrSbII-50, AAO61094
I8_2
Desulfobacter vibrioformis, AJ250472
Desulfohalobium retbaense, AF418190
Desulfonatronovibrio hydrogenovorans, AF418197
Desulfomicrobium apsheronum, AB061529
Desulfonatronum lacustre, AF418189
marine sediment clone NKeSL1, ACH73209
I61_21 (2 clones)
uranium-mining site clone P4D-11, AAV98376
III_60
Desulfomonile tiedjei, AF334595
leachate-polluted aquifer clone LGWN07, ABK90811
II28_36 (9 clones)
leachate-polluted aquifer clone LGWG21, ABK90693
deep-sea sediment clone NTd-VII01, BAE96525
I38_15 (4 clones)
III_3
leachate-polluted aquifer clone LGWI04, ABK90733
leachate-polluted aquifer clone LGWI12, ABK90739
I83_11 (4 clones)
Desulfofustis glycolicus, AF418191
Desulfonhopalus singaporensis, AF418196
Desulfotalea arctica, AY626032
Desulfotalea psychrophila LSv54, NC_006138
Desulfonhopalus vacuolatus, AF334594
Desulfobulbus propionicus, AF218452
Desulfobulbus elongatus, AF418202
Desulfobulbus rhabdoformis, AJ250473
polluted harbor sediment clone VHS057, AAZ15766
III_2
Syntrophobacter wolinii, AF418192
Syntrophobacter fumaroxidans, AF418193
hot spring clone YSdsr4, BAB86795
III_23
deep-sea sediment clone NTd-II04, BAE96473
I55_19 (2 clones)
leachate-polluted aquifer clone LGWK18, ABK90765
III_43
freshwater clone O14, BAF42756
II44_43 (3 clones)
leachate-polluted aquifer clone LGWG25, ABK90667
III_93
leachate-polluted aquifer clone LGWG08, ABK90671
leachate-polluted aquifer clone LGWI10, ABK90713
II6_25 (5 clones)
acidic fen clone 3_20, CAJ47265
III_16
acidic fen clone dsrSbII-15, AAO61108
III_15
I49_17
Desulfobacca acetoxidans, AF482453
III_18
III_25
wetland clone F1SU, AAM21737
marine sediment clone 25H-0D-71, ACJ11323
III_74
Desulfotomaculum reducens MI-1, NC_009253
Desulfotomaculum ruminis, U58118 U58119
Desulfotomaculum nigrificans, AF482466
Desulfotomaculum alkaliphilum, AF418195
Desulfotomaculum halophilum, AY626024
freshwater clone B36, BAF42729
III_35
II55_22 (10 clones)
acidic fen clone, CAJ47300
III_10
Thermodesulfovibrio islandicus, AF334599
0.10
Desulfobacterales
Clostridiales
Desulfobacterales
Desulfovibrionales
Syntrophobacterales
Desulfobacterales
Syntrophobacterales
Clostridiales

Figure 4

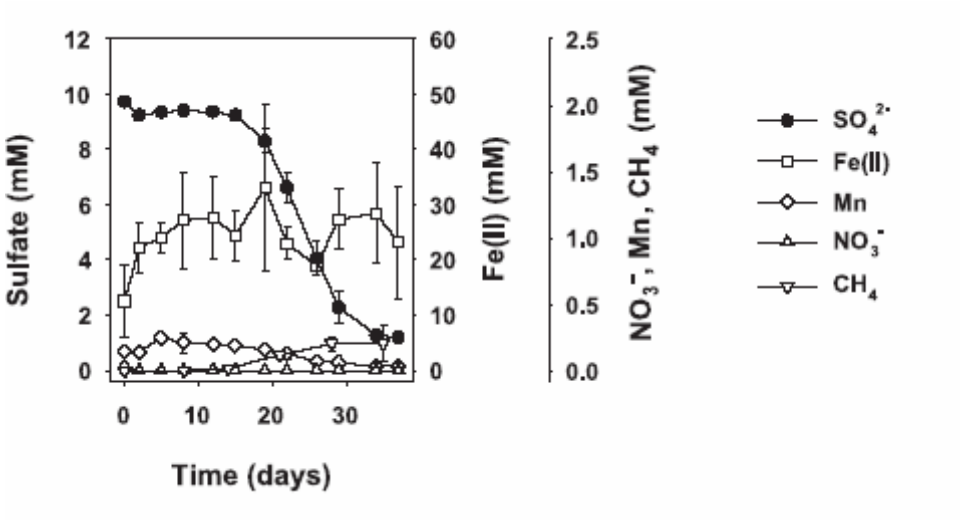

Figure 5

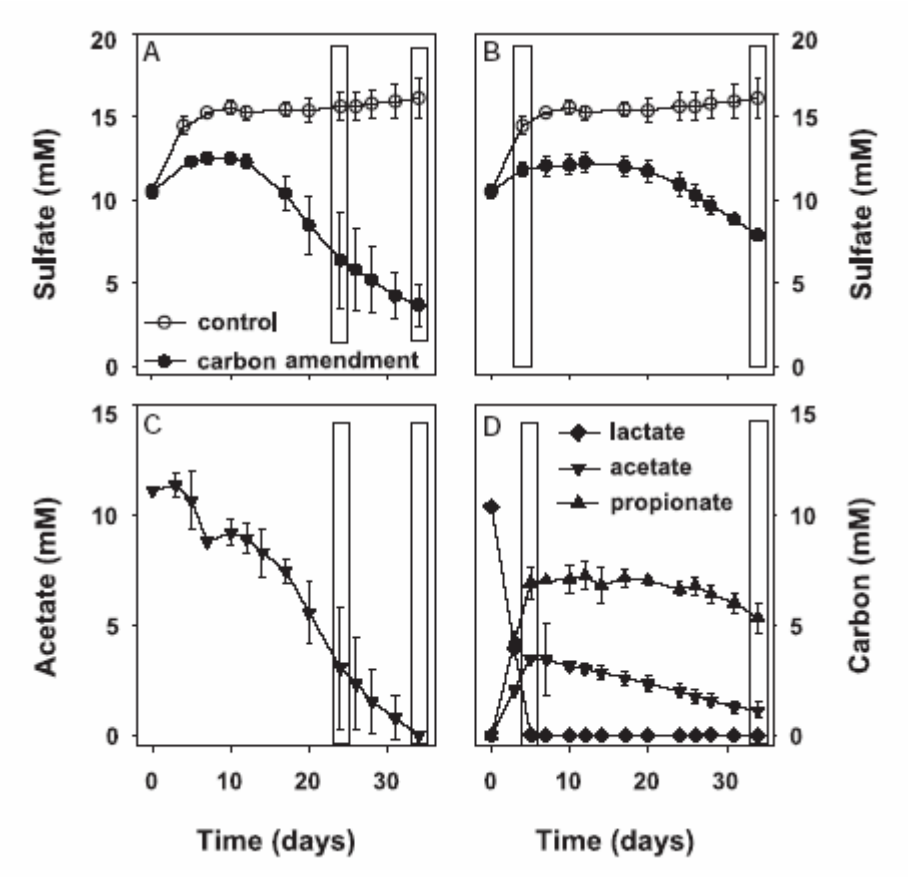
A
B
C
D
Sulfate (mM)
Acetate (mM)
Carbon (mM)
control
carbon amendment
lactate
acetate
propionate
Time (days)
Time (days)
0
5
10
15
20
0
10
20
30

Figure 6

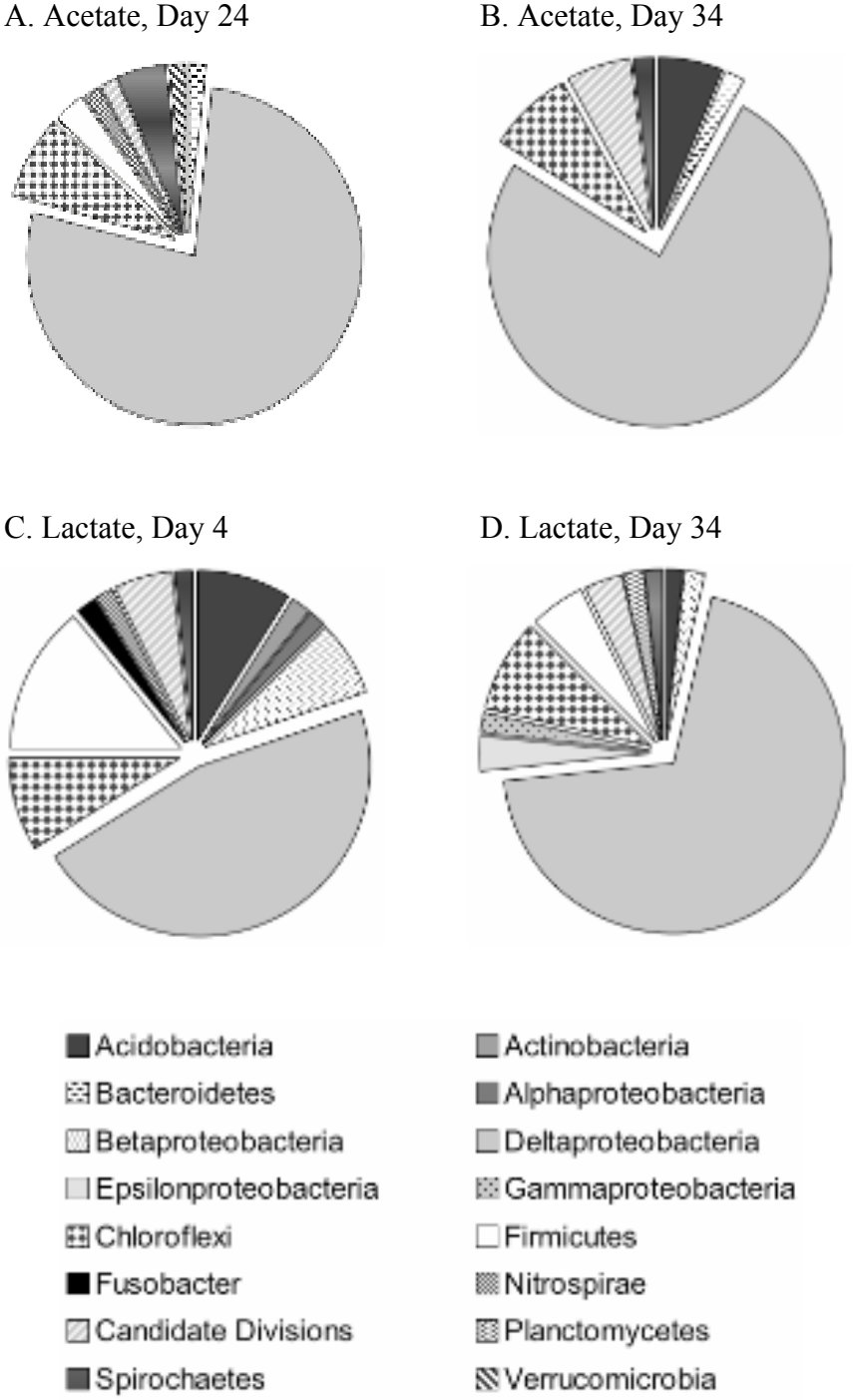
A. Acetate, Day 24
B. Acetate, Day 34
C. Lactate, Day 4
D. Lactate, Day 34
Acidobacteria
Actinobacteria
Bacteroidetes
Alphaproteobacteria
Betaproteobacteria
Deltaproteobacteria
Epsilonproteobacteria
Gammaproteobacteria
Chloroflexi
Firmicutes
Fusobacter
Nitrospirae
Candidate Divisions
Planctomycetes
Spirochaetes
Verrucomicrobia

Table 1: Total metal content of Br1 and Br2 solid phase.

Horizon	Metal content (µg g [dry wt soil]$^{-1}$)[a]						
	U	Zn	Ni	Cu	Co	As	Cd
Br1	343 ± 4	425 ± 10	170 ± 1	289 ± 2	31 ± 1	37 ± 1	5 ± 0
Br2	959 ± 3	565 ± 11	229 ± 1	325 ± 3	43 ± 0	55 ± 1	5 ± 0
BBodSchV[b]	n.d.[d]	60 - 200	15 - 70	20 - 60	n.d.[d]	25	0.4 - 1.5
IAEA[c]	2	n.d.[d]	n.d.[d]	n.d.[d]	n.d.[d]	n.d.[d]	n.d.[d]

[a] Values represent metal content in µg g^{-1} dry weight of Br1 or Br2 soil and background soil levels.

[b] Bundes-Bodenschutz- und Altlastenverordnung; http://www.gesetze-im-internet.de/bundesrecht/bbodschv/gesamt.pdf (accessed 30 Nov 2009). BBodSchV values range from limits in sand to limits in clay material.

[c] International Atomic Energy Agency; http://www.iaea.org/NewsCenter/Features/DU/du_qaa.shtml (accessed 30 Nov 2009). Background soil level is given.

[d] Standard values are not given.

Table 2: Enumeration of sulfate-reducing bacteria in reduced soil horizons in the bank of the Gessen Creek with and without metals.

Treatment	Electron donor	MPN (cells g [fresh wt soil]$^{-1}$)[a]	
		Br1	Br2
without metals	acetate	2.0×10^4 (0.4×10^4 - 9.3×10^4)[b]	9.3×10^3 (1.9×10^3 - 4.2×10^4)[b]
without metals	lactate	2.0×10^4 (0.4×10^4 - 9.3×10^4)[b]	7.0×10^5 (1.5×10^5 - 3.3×10^6)[b]
with metals	acetate	4.0×10^4 (0.8×10^4 - 1.9×10^5)[b]	4.0×10^3 (0.8×10^3 - 1.9×10^4)[b]
with metals	lactate	2.0×10^4 (0.4×10^4- 9.3×10^4)[b]	4.0×10^4 (0.8×10^4 - 1.9×10^5)[c]

[a] Values represent abundances in cells g (fresh weight of Br1 or Br2 soil)$^{-1}$, determined in triplicate MPN serial dilutions after 5 months of incubation at 16°C. Values in parentheses represent the range of MPN values within 95% certainty.

[b,c] Significant differences of MPN values between metal treatments for the different soil horizons and electron donors.

Table 3: Soluble metal concentration at the beginning (T_0) and end (T_{end}) of incubation of Br1 and Br2 soil suspension.

Experiment		Soluble metal concentration (nM)[a]											
		U		Ni		Co		Zn		Cu		As	
		Ctr	Ace	Ctr	Ace	Ctr	Ace	Ctr	Ace	Ctr	Ace	Ctr	Ace
Br1	T_0	9 ± 3	14 ± 8	538 ± 393	508 ± 215	188 ± 128	194 ± 47	266 ± 79	196 ± 31	22 ± 6	17 ± 6	520 ± 46	365 ± 46
	T_{end}	35 ± 13	148 ± 48	91 ± 19	40 ± 8	32 ± 8	8 ± 0	118 ± 23	78 ± 9	5 ± 4	26 ± 3	498 ± 20	190 ± 57
Br2	T_0	1 ± 0	1 ± 0	422 ± 46	422 ± 46	92 ± 8	92 ± 8	277 ± 309	277 ± 309	5 ± 2	5 ± 2	417 ± 30	417 ± 30
	T_{end}	9 ± 2	256 ± 60	71 ± 10	42 ± 4	18 ± 1	6 ± 1	53 ± 14	36 ± 36	10 ± 2	16 ± 2	1444 ± 38	1629 ± 76
Br2 (SIP)[c]	T_0	6 ± 1	6 ± 1	1210 ± 402	1210 ± 402	133 ± 67	133 ± 67	34 ± 33	34 ± 33	31 ± 0	31 ± 0	343 ± 37	343 ± 37
	T_{end}	8 ± 1	1407 ± 332	839 ± 156	69 ± 6	74 ± 8	9 ± 1	234 ± 102	15 ± 0	31 ± 0	85 ± 15	248 ± 18	560 ± 81

[a] Values represent soluble average metal concentration in nM of Br1 or Br2 soil suspension at the beginning (T_0) and end (T_{end}) of the microcosm experiments (n=3) treated as a control (Ctr) or amended with acetate (Ace).

[b] below detection (b.d.)

[c] second experiment with soil of Br2 horizon

Metal tolerance of Fe(III)-reducing microbial communities in a contaminated creek soil

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Abstract

Fe(III)-reducing microorganisms facilitate the release of sorbed metals from Fe(III)-minerals by reductive dissolution, and therefore metal concentrations in their surrounding environment are enhanced. Their tolerance towards high metal concentrations might be important for metal mobilization processes in soils. However, until know only little is known about metal tolerance of Fe(III)-reducing microorganisms. The aim of this study was to estimate the effect of microbial Fe(III)-reduction on metal mobility and on the stability of metal reposition in a contaminated soil. Metal tolerance of indigenous Fe(III)-reducers was determined, the microbial communities were analyzed by DGGE, cloning, and sequencing, and the *in situ* abundance of *Geobacteraceae* was investigated by quantitative PCR. Geochemical data indicated the presence of Fe(III)-reduction in an iron- and metal-rich horizon of a gleyic creek soil, but Fe(II)-formation rates were low without biostimulation. Generally, only µmolar metal concentrations were tolerated and Fe(III)-reduction might be inhibited by soil concentrations of Cu and Ni. In a mixed clone library of metal amended cultures almost exclusively *Geobacter*-related *δ-Proteobacteria* and *Firmicutes* were present. Sequencing of DGGE bands demonstrated a dominance of *Firmicutes* in Zn-tolerant cultures, suggesting an importance of *Firmicutes* for Fe(III)-reduction in contaminated environments. An isolate related to *Firmicutes* was obtained from Cu- and Cd-amended cultures. In soil DNA samples 10^6 *Geobacteraceae* genomes per g fresh weight soil were detected. Our results suggested that microbial Fe(III)-reduction in soils might be inhibited already at low metal concentrations and might depend on a metal scavenging process or a sufficient carbon source to fuel detoxification.

Introduction

Although many metals are important micronutrients they are toxic for organisms at high concentrations (Bruins et al., 2000). In soils a high proportion of metals can be immobilized on surface sorption sites of Fe(III)-minerals or be included in the mineral structure (Schwertmann and Taylor 1989, Bradl 2004). Fe(III)-reducing bacteria (FeRB) can facilitate the release of these metals by reductive dissolution of Fe(III)-oxides (Francis and Dodge 1990, Cambier and Charlatchka 1999). *Shewanella putrefaciens* can release bioreduced Co from Fe(III)-oxides during microbial Fe(III)-reduction (Zachara et al., 2001). Thus, FeRB might enhance metal availability in their surrounding environment and are therefore exposed to high metal stress. This implies that metal tolerance should be an important attribute for FeRB.

FeRB are in discussion for use in bioremediation approaches, like the cleanup of radionuclide contaminated sites (Wilkins et al. 2006), and a high metal tolerance might be necessary to grow in such contaminated soils. However, in contrast to some well investigated metal-resistant model organisms like *Cupriavidus metallidurans* (Nies 2003), there is only little known about the metal tolerance of FeRB. Studies with *Shewanella oneidensis* demonstrated tolerances of only µM concentrations of Cd, Co, Cu, and Zn (Francis and Dodge 1988, Toes et al. 2008). The acidophilic FeRB *Acidiphilium cryptum* tolerates mM concentrations of Cd, Cu, Ni, and Zn (Dopson et al. 2003). However, metal solubility is much higher at acidic than at neutral pH. Metal tolerances of acidophilic and neutrophilic organisms are therefore not comparable. For genus *Geobacter* no metal tolerances are known to our knowledge, although it has been shown that during growth in sediments originating from a uranium contaminated site some proteins are expressed which might be connected to metal resistance (Holmes et al. 2009).

In the former mining district Ronneburg (Thuringia, Germany), one of the most productive mining districts in the former German Democratic Republic, uranium mining caused severe environmental contaminations with radionuclides and a variety of heavy metals (Jakubick et al., 1997). The creek Gessenbach was one of the main drainage systems and received metal containing seepage waters and surface runoff from upstream mining sites (Wismut 2006a). Contaminant inflow strongly declined over the last years as a result of remediation (Wismut 2006a, Wismut 2006b) but contaminants enriched over the last decades remain in the soil. In a previous study we demonstrated the solubilization of Co, Ni, Zn, As, and U during biostimulated microbial Fe(III)-reduction in soil microcosms (Burkhardt et al. 2010). This process was associated with the activity of microorganisms related to *δ*-

Proteobacteria, *Acidobacteria*, *Firmicutes*, and *β-Proteobacteria*. However, without biostimulation no or very low Fe(III)-reduction could be observed, indicating an energy demanding metal-resistance mechanism.

The aim of this study was to estimate the effect of microbial Fe(III)-reduction on metal mobility and on the stability of metal reposition in a contaminated soil. For this, metal tolerance of indigenous Fe(III)-reducers was analyzed. The microorganisms most likely involved in *in situ* Fe(III)-reduction were determined using *in situ* quantification of *Geobacteraceae* and identification of microorganisms in Fe(III)-reducing metal amended enrichment cultures. The diversity and variability of microbial communities was characterized by DGGE in the whole soil profile.

Materials and Methods

Study site and sampling. The sampling site is located in the former uranium mining district Ronneburg (Germany) at the bank of the creek Gessenbach in a gleyic creek soil. The soil profile sampled has been described before (Küsel et al. 2008, Burkhardt et al., 2010, Sitte et al. submitted). Shortly, this profile consists of two upper horizons (Ah, BEw/Ah), two groundwater influenced oxidized horizons (BElc, Btlc), and two groundwater influenced reduced horizons (Br1, Br2). High concentrations of a variety of metals were detected in particular in horizons Btlc and Br2 and pore water metal concentrations peaked in horizon Btlc. Horizon Btlc has the highest iron content of the soil profile.

Pore water samples were taken monthly from June to November 2007 as described previously (Burkhardt et al. 2010). Soil samples for sequential extraction or DNA extraction were collected from all horizons in August 2006 or June 2007, respectively, and were stored at -20°C until DNA extraction. Btlc samples for MPN-analysis and cultivation of metal tolerant organisms were taken in June 2007 or April 2008. Until start of the cultivation samples were stored at 4°C for 1 – 3 months.

Analytical techniques and rate calculation. Iron(II) and total Fe were measured after HCl extraction with the phenantroline-method (Tamura et al., 1974) as described previously (Küsel et al., 2002; Burkhardt et al., 2009). Nitrate was measured as described by Velghe and Claeys (1985). Dissolved metals and As were measured in filtered (0.45 µM nylon filters) culture samples acidified with nitric acid (65%, Roth, Karlsruhe, Germany) with ICP-MS (inductively coupled plasma – mass spectrometry; X-Series II, Quadrupol ICP-MS, Fa.

Thermo Electron, Bremen). Each sample was measured 4 times. Binding forms of elements were determined by sequential extraction using the method of Zeien and Brümmer (1989) as described previously (Burkhardt et al., 2009, Grawunder et al., 2009). Shortly, the method yielded the following 7 fractions which can be correlated to putative binding forms: mobile (fraction 1), specifically adsorbed (fraction 2), elements bound to manganese oxides (fraction 3), elements bound to organic material (fraction 4), elements bound to amorphous iron oxides (fraction 5), elements bound to crystalline iron oxides (fraction 6), elements bound to the residual fraction, presumably mainly silicates (fraction 7). Fe(III)-reduction rates were calculated for soil microcosms, which have been described previously (Burkhardt et al., 2010, Sitte et al., in press). Fe(II)-formation, measured as described above, was used as a measure for Fe(III)-reduction. Rates were calculated as the regression slope of the period of linear increase of Fe(II)-concentrations.

Quantification of microorganisms. The abundance of *Geobacteraceae* in soil DNA samples was analyzed by quantitative PCR. DNA was extracted from soil samples using the UltraClean PowerSoil DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA, USA) applying the alternative lysis method as given by the manufacturer. The 16S rRNA gene was amplified from 10 ng genomic DNA using the primers 561F (5´-GCGTGTAGGCGGTTTCTTAA-3´; Stults et al., 2001) and Geo858R (5´-CTAGGTGTTGCGGGTATTGA-3´; this study) and the reaction mix SensiMix^{TM} *LowRef* (Quantace, distributed by Bioline GmbH, Luckenwalde, Germany) in the following temperature program: 10 min 95°C, 50 x (15 sec 95°C, 30 sec 61°C, 30 sec 72°C, 10 sec 78°C) with fluorescence measurements at 61, 72, and 78°C, followed by a dissociation curve analysis with 1 min 95°C, 30 sec 55°C, and heating to 95°C with a rate of 0.01°C sec^{-1}. Genomic DNA from pure culture *Geobacter sulfurreducens* (DSM 12127) was used as standard. Genome copies were calculated based on the molecular weight of the genome of *G. sulfurreducens*. Primer Geo858R was designed using the ARB Primer Design module against an alignment of *Geobacter* sequences (Ludwig et al., 2004) and was tested for its specificity with organisms of different phylogenetic affiliation (table 1). PCR conditions were optimized for *G. sulfurreducens* DNA, ensuring minimal yield for DNA of *Desulfuromusa bakii*, *Pseudomonas fluorescens*, and *Geothrix fermentans*. Reactions that yielded Ct-values with less than three cycles difference to the non-template control were considered to be within the error of the measurement. Thus, the results for *Desulfuromusa bakii*, *Clostridium glycolicum*,

and *Sporomusa rhizae* were considered to be negative. The reaction yielded between 10^2 and 10^7 genome copies for different *Geobacter* species. Low copy numbers of 10^2 to 10^3 were detected for *Geobacter* FRC-32, *Geobacter metallireducens*, and *Geobacter pelophilus*.

For statistical analysis of *Geobacter* genome copy numbers a one-way analysis of variance (ANOVA) with Bonferroni post-hoc test was performed on transformed values (log10(value+1)) using SPSS 15.0 (SPSS Inc., Chicago, Illinois, USA). The level of significance was 0.05. Normal distribution and homogeneity of variances were suggested by the outcome of a Kolmogorov-Smirnov test and Levene´s test, respectively.

Culturable Fe(III)-reducing microorganisms were enumerated in Btlc soil using a most probable number (MPN) approach in the absence or presence of additional heavy metals. Metal concentrations were added to a final concentration of 150 µM Co, 10 µM Cr, 1.5 mM Cu, 1.0 mM Ni, 2.5 mM Zn, 30 µM Cd, and 20 µM Pb in the media to meet the bioavailable solid phase concentrations as detected by sequential extraction. Tenfold serial dilutions of Btlc soil were prepared in sterile anoxic 0.7% NaCl. For each dilution and treatment, 3 replicates were inoculated into anoxic growth media consisting of (per liter) 50 mg yeast extract, 5 mL trace metal solution (Drake, 1994) and 5 mL vitamin B solution (Drake, 1994). As electron acceptor, 50 mM goethite, prepared according to Cornell and Schwertmann (2003), was added. The pH was adjusted to 5.2 and glucose or lactate was added separately to reach a final concentration of 5 mM. The cultures were incubated in the dark at 15°C and growth was observed by Fe(II)-measurements as described above. The number of culturable cells, 95% confidence intervals, and significance of differences were estimated from standard MPN tables Alef (1991).

Cultivation of Fe(III)-reducers in the presence of heavy metals. Fe(III)-reducers were enriched in a basal medium (Lin et al., 2007) with each 1 mM ethanol and lactate as carbon source. As electron acceptor 14 mM $Fe(OH)_3$, prepared according to Lovley and Phillips (1986) and washed 4 times with bidistilled water, were added to the medium. $FeCl_2$ (0.5 mM final concentration) was added as a reductant. Four concentrations of each metal (Cd, Cu, Ni, Zn) were added to the culture tubes (table 1). Metal concentrations were chosen based on pore water and bioavailable solid phase metal concentrations of the sampled soil horizon (Küsel et al., 2008, Burkhardt et al., 2009). Also, tubes were prepared with mixes of all metals representing pore water concentrations (mix 1) and bioavailable solid phase concentrations (mix 2). For each metal treatment triplicate cultures were inoculated with 1 mL soil

suspension (9 mL 0.7% NaCl + 1 g soil) in 9 mL medium. Additionally, a sterile control, where only 0.7% NaCl was added, was prepared for each treatment. One set of cultures was left without metal amendment. The final pH of the cultures was 6.5 - 6.8 (pH-meter pH330, WTW, Weilheim, Germany, with a micro-pH-electrode InLab 423, Mettler-Toledo, Gießen, Germany). Cultures were incubated statically at 15°C in the dark and the activity of Fe(III)-reducing microorganisms was monitored regularly by measuring Fe(II)-formation. At the end of the experiment dissolved metal concentrations were measured as described above in selected metal amended cultures and the corresponding sterile controls to check for precipitation. Zn was detected with ICP-OES (inductively coupled plasma – optical emission spectrometry; Spectroflame P FAV05, Spectro Analytical Instruments, Kleve). Based on the results of this first experiment metal concentrations were increased or intermediate concentrations were chosen for a second experiment. Additionally, the metalloid As was tested. The second experiment was conducted in the same way as the first. For both experiments the same soil sample was used for inoculation and was stored at 4°C in between.

DNA extraction and clone library construction from metal containing cultures. To identify metal tolerant FeRB, three cultures (amended with 1.1 µM Cu, 150 µM Ni, or 2500 µM Zn) were chosen for clone library construction. These cultures each contained the highest concentrations tested for one metal that still allowed Fe(II)-formation. Culture samples were centrifuged (13.780 g, 15 min) and DNA was extracted from the pellets as described above. For PCR amplification the general domain *Bacteria* SSU rRNA gene primers fD1 (5'-AGA GTT TGA TCC TGG CTC AG-3') and rP2 (5'-ACG GCT ACC TTG TTA CGA CTT-3', both Weisburg et al. 1991) and the FailSafe PCR PreMix D (EPICENTRE Biotechnologies, Madison, WI, USA, provided by Biozym Scientific GmbH, Hess. Oldendorf, Germany) were used. PCR products were gel-purified (Agarose Gel Extraction Kit, Jena Bioscience, Jena, Germany). During gel-purification PCR products of all three DNA extracts were combined. The cleaned PCR products were used to construct a clone library using the pGEM-T Vector System and JM109 High Efficiency Competent Cells (both Promega, Madison, USA) following the manufacturers instructions. The cloned sequences were reamplified using the vector specific primers M13 forward and M13 reverse as given by the manufacturer. Restriction fragment length polymorphism (RFLP) analysis was used to group the clones into phylotypes based on pattern similarity after cutting with the restriction enzymes *Bsh*F1 and *Msp*I (both Fermentas, St. Leon-Rot, Germany). Representative clones from each phylotype

were sequenced bidirectionally (AGOWA GmbH, Berlin, Germany). Sequences were assembled using BioEdit version 7.0.5.3 (Hall, 1999). Prior to comparative phylogenetic analysis, vector and primer sequences flanking the SSU rRNA gene inserts were removed. Previously identified sequences with high sequence similarity to the clones obtained in this study were determined using the BLAST algorithm against the GenBank database available from National Center for Biotechnology Information (NCBI) (Altschul et al. 1990). Clone sequences were checked for chimeras using the program Chimera Check from the Ribosomal Database Project II (Cole et al. 2003), the Bellerophon server (Huber et al., 2004, accessible at http://foo.maths.uq.edu.au/~huber/bellerophon.pl), and Pintail (Ashelford, 2007, accessible at http://www.bioinformatics-toolkit.org/Web-Pintail/). Clones were grouped into phylotypes based on a sequence similarity cut off of 97% using FastGroupII (Yu et al., 2006). Statistical analyses were used to determine the sampling efficiencies and coverage of the clone library (Good 1953; Singleton et al. 2001).

Denaturing gradient gel electrophoresis (DGGE). DGGE was used to compare community fingerprints of the metal tolerant cultures, the representative clones, and the microbial community present in the soil. For this, the same DNA extracts as for qPCR and clone library construction were used. The DNA was amplified using the general domain *Bacteria* SSU rRNA gene primers mentioned above and the FailSafe PCR PreMix C (EPICENTRE Biotechnologies, Madison, WI, USA, provided by Biozym Scientific GmbH, Hess. Oldendorf, Germany) in the following temperature program: 5 min 95°C, 30 x (1 min 95°C, 1 min 56°C, 1 min 72°C), 10 min 72°C. The PCR-products from this reaction were used for nested PCR in a similar temperature program as before with only 20 cycles and 54°C annealing temperature. For nested PCR, the primers R1401 and F968GC (Kozdrój and van Elsas, 2000) and the FailSafe PCR PreMix G (EPICENTRE Biotechnologies, Madison, WI, USA, provided by Biozym Scientific GmbH, Hess. Oldendorf, Germany) were used. The nested PCR products were gel quantified using the software Gene Tools 4.0 (SynGene, Cambridge, England) and 50ng, 500ng, or 800ng nested PCR product were loaded on the DGGE gel for clone samples, culture samples, or soil samples, respectively. Gradient gels contained 8% acrylamide and the gradient ranged from 50% to 65%. DGGE was run at 60°C and 100 V for 15 hours in 0.5x TAE buffer (20 mM Tris, 0.5 mM EDTA, pH 8). Gels were stained with SybrGold (Invitrogen, Carlsbad, CA USA) for documentation. Analysis of band patterns and intensities was also done with the Gene Tools 4.0 software. For comparison of band patterns a cluster

analysis was done by unweighted pair group method with arithmetic mean (UPGMA) with Pearson coefficient using DendroUPGMA (accessible at http://genomes.urv.cat/UPGMA/).

Isolation of a heavy metal tolerant FeRB. An FeRB was isolated from an FeRB enrichment culture tolerant to 0.1 mM Cd or Cu using a modified agar shake technique. Agar shake media contained (per liter) 1.0 g NaCl, 0.4 g of $MgCl_2*6H_2O$, 0.1 g of $CaCl_2*H_2O$, 0.5 g KCl, 0.3 g of NH_4Cl and 0.6 g of KH_2PO_4, 20 mM $Fe(OH)_3$, 50 mM HEPES, 100 µM AQDS, 0.5 mM $FeCl_2$, 0.5% agar (AppliChem), 1 ml vitamin solution M141 (Benz et al. 1998), 1 ml trace element solution SL9 (Benz et al. 1998), and 1 mM each lactate and ethanol. Agar shake cultures were incubated at 22ºC in the dark for 3 weeks, when distinct black colonies formed. Colonies were picked using sterile, anaerobic technique and transferred to fresh agar shakes. After 3 subsequent transfers in agar shake media colonies were transferred to the liquid media described above without agar and with 0.1% yeast extract and 30 mM bicarbonate as a buffer. Growth, Fe(III)-reduction, electron donor utilization and yeast-dependent growth was evaluated in liquid media using HPLC and measuring HCl extractable iron as described below.

DNA was extracted from isolate DA-1 by boiling a colony in 100 µl 5% (w/v) Chelex 100 Resin (Bio-Rad) for 10 min. The sample was centrifuged and 2 µl of the supernatant added to a 50 µl PCR reaction for amplification with 16S rRNA gene primers fD1 and rP2 (see above). Isolate DA-1 was sequenced completely (AGOWA GmbH, Berlin, Germany) and assembled using Geneious Pro version 4.6.4 (Biomatters, http://www.geneious.com).

Results

Geochemistry of the soil profile. Pore water geochemistry was comparable at all sampling times. Major changes occurred in horizon Btlc. Nitrate concentrations were variable in the upper horizons, decreased with depth, and were completely depleted within horizon Btlc (figure 1). Fe(II) is accumulating in the lower soil horizons, starting in the lower part of horizon Btlc (figure 1). Several metals peaked in the Btlc-horizon (Burkhardt et al. 2010, Sitte et al. submitted). The maximum metal concentrations detected in Btlc pore water are summarized in table 2. Our data suggested, that Btlc was a redox transition zone were metal solubility might be altered by microbial activities. Due to the high iron-content and the beginning Fe(II)-accumulation in horizon Btlc, Fe(III)-reduction is likely to occur and trigger metal release in this horizon.

Sequential extraction data indicated that 20 to 40% of most metals, and even 80% of As, were bound to iron oxides, in particular to amorphous iron oxides, in horizon Btlc (figure 2), and might potentially be released by reductive dissolution of Fe(III)-minerals. Another important metal sorbent was silicate, which is represented by the residual fraction. The fractions of metals that are mobile, replenishable, or bound to manganese oxides or organic matter were important only for few metals.

Community fingerprints of the soil horizons. Microbial community fingerprints clearly differed between all soil horizons (figure 3). While some bands were present in several horizons, also horizon-specific bands could be detected. The patterns grouped into two distinct clusters separating the reduced horizons Br1 and Br2 from the oxidized horizons Ah/BEw, BElc and Btlc. Within the cluster of oxidized horizons the horizons BElc and Btlc grouped together. Variability between patterns of triplicate samples was greatest for horizon Ah/BEw and lowest for Br1 and Br2. Compared with the reduced horizons variability in BElc and Btlc was relatively high, probably reflecting greater small scale variability.

Fe(III)-reduction rates. Despite the high Fe(III) and carbon content of the Btlc horizon, Fe(III)-reduction was low to negligible without biostimulation (figure 4, Burkhardt et al., 2009). Biostimulation increased Fe(III)-reduction rates up to four times. In the deeper soil horizons Br1 and Br2, Fe(III)-reduction rates similar to biostimulated Btlc soil were observed independently of carbon amendment. Fe(II)-formation ceased at about 30 or 40 mM in Br1 or Br2 soil, respectively, and no Fe(III)-reduction could be observed if Fe(II) concentrations were already in this range at the beginning of incubation. In Btlc soil Fe(II) concentrations were not higher than 4 mM at the beginning of incubation, and up to 30 mM Fe(II) were formed in biostimulated soil microcosms until the end of the experiments, demonstrating the potential for Fe(III)-reduction in this horizon. Nonetheless, unlike in Br1 and Br2 soil, Fe(III)-reduction in Btlc soil was inhibited without biostimulation.

Quantification of Fe(III)-reducers in the soil horizons. With quantitative PCR *Geobacter* genome copies in the order of 10^6 per gram soil [ww] were detected in the soil horizons BElc, Btlc, Br1, and Br2 (Table 3). In general, *Geobacter* abundance decreased with depth, and a significantly higher copy number per gram soil [ww] was observed in DNA extracts from horizon BElc, while no significant difference was observed between *Geobacter* copy numbers

of Btlc, Br1, and Br2 soil DNA samples. Nonetheless, copy numbers in all horizons were in the same order of magnitude, indicating that *Geobacter* abundance was not substantially decreased by metal contaminants in horizon Btlc.

MPN numbers of culturable Fe(III)-reducing microorganisms reached 10^4 cells/g Btlc soil [ww] for both carbon sources used if no metals were added to the cultures (Table 4). The presence of metals significantly inhibited Fe(III)-reduction and only 10^1 cells/g Btlc soil [ww] of metal tolerant Fe(III)-reducers were detected. The metal concentrations added corresponded to the potentially bioavailable mobile and replenishable fractions of soil solid phase sequential extraction, indicating the possibility of inhibition of Fe(III)-reduction by in-situ metal concentrations.

Metal tolerance of Fe(III)-reducing enrichment cultures. Within the concentration range tested in the first experiment toxic effects were observed for Cu, Ni, Zn, and Co. Toxic effects were indicated by a reduction of the Fe(II)-formation-rates and/or an elongation of the lag phase (Table 5). In a second experiment metal tolerance of Fe(III)-reducers could be further specified for Cd, Cu, Ni, Al, Pb, and, additionally, As (data not shown). For Co and Cr no higher concentrations could be tested, as both metals interacted with the Fe(II)-measurement at high concentrations (Vydra and Přibil 1960, Kuwabara et al. 1999).

The addition of 150 µM Cu caused a complete inhibition of Fe(II)-formation, while it was not affected by 1.1 µM Cu. In the second experiment Fe(II)-formation was slowed but not completely inhibited in the presence of 100 µM Cu. Fe(II)-formation rates were reduced by 150 µM Ni, but not by 20 µM Ni. A concentration of 1000 µM Ni completely inhibited Fe(II)-formation. 600 µM Ni slowed, but did not completely inhibit Fe(II)-formation in the second experiment. In the Zn-amended cultures Fe(II)-formation was reduced and the lag phase was extended by the addition of 2500 µM, but not 500 µM Zn. Al was tolerated by Fe(III)-reducers at all concentrations tested and in the second experiment no inhibition of Fe(II)-formation was observed up to 10 mM Al. The addition of Co did not cause a decrease in Fe(II)-formation rates although the lag phase was elongated in cultures amended with 150 µM Co. For the Cd treatments data indicated a decrease in Fe(II)-formation rates. The lag phases in the Cd amended cultures did not change within the concentration range tested. In the second experiment, the presence of 100 µM Cd was still tolerated by Fe(III)-reducers, but at 500 µM Cd no Fe(II)-formation could be observed any more. For Cr and Pb no toxic effects could be observed up to 100 µM. In the second experiment no inhibition of Fe(II)-formation

could be observed up to 1000 µM Pb. As did not inhibit Fe(II)-formation up to a concentration of 800 µM, but our results indicated a slight inhibition at 1600 µM As.

At the end of the experiment, dissolved metal concentrations were measured in some metal tolerant cultures and the corresponding sterile controls to check for sorption or precipitation. If 150 µM Ni, 2500 µM Zn, 1.1 µM Cu, 100 µM Cu, or 100 µM Cd were added to the cultures 73 µM Ni, 114 µM Zn, 0.8 µM Cu, 23 µM Cu, or 14 µM Cd were dissolved in sterile controls, respectively. In the inoculated cultures concentrations were even lower (35 µM Ni, 35 µM Zn, 0.02 µM Cu, 0.01 µM Cu, and 2.0 µM Cd, respectively), probably due to metal sorption to cell surfaces. These results indicate that metal tolerance was even lower than suggested by total metal concentrations tolerated by Fe(III)-reducing enrichment cultures.

Pore water concentrations of all metals, added separately or as a mix (mix 1), were tolerated by Fe(III)-reducing cultures, also if dissolved metal concentrations were considered. A mix of all metals tested in concentrations equalling the bioavailable fraction of the soil solid phase (mix 2) completely inhibited Fe(III)-reduction. At these concentrations, only Cu and Ni completely inhibited Fe(III)-reduction when amended separately and minor inhibitive effects were observed for separate amendments of Zn, Co, and Cd, suggesting that Ni and Cu were the main inhibitive contaminant metals in horizon Btlc.

Identification and isolation of metal tolerant microorganisms. A mixed clone library of three metal containing cultures (1.1 µM Cu, 150 µM Ni, 2500 µM Zn) was composed almost exclusively of *δ-Proteobacteria* (62%) and *Firmicutes* (36%, table 6). Only one clone could be assigned to *β-Proteobacteria*. The *δ-Proteobacteria* clones were dominated by clones related to *Geobacter psychrophilus*, while such a dominance of a single organism did not occur in the *Firmicutes* group. The coverage of the clone library was 0.94.

DGGE patterns of metal tolerant enrichment cultures showed much less bands than patterns of soil sample DNA (figure 5). The main bands of the metal amended cultures matched only weak bands or were not detected in the soil DGGE patterns. It was not possible to identify organisms in the DGGE patterns of soil or cultures by comparison with DGGE patterns of clones, as clones of different phylogenetic affiliation had similar band positions and some clones yielded multiple bands (data not shown). However, by sequencing the three main DGGE bands of the culture amended with 2500µM Zn, all three bands could be identified as *Firmicutes* (figure 5).

A strain, DA-1, affiliated with *Firmicutes* was isolated from enrichment cultures amended with 100 µM Cd or Cu. Strain DA-1 was most closely related to the uncultured bacterium clone GIF4 [AF407196.1] and *Desulfosporosinus lacus* strain STP12T [AJ582757.1] (both 98% sequence similarity).

Discussion

In-situ geochemistry and microbiology. Peaks in pore water metal concetrations indicate the release of metals from Fe(III)-oxides in horizon Btlc, probably by reductive dissolution, as it has been observed before in soil microcosm studies (Burkhardt et al. 2010). However, Fe(III)-reduction was inhibited in Btlc-soil without biostimulation. Only if a carbon source was added, Fe(III)-reduction rates were similar to those measured in horizon Br1 and Br2. An additional energy source seems to be necessary to drive energy consuming detoxification processes, like ATP-driven efflux pumps (Silver, 1996). Alternatively, the metals detected in the Btlc-horizon might also be transported to this site by seepage water.

Microbial communities were clearly different between the oxidized and reduced horizons of the soil profile, which was expected based on their different geochemistry (Burkhardt et al., 2009, Sitte et al., in preparation). In the oxidized horizons geochemical conditions indicated nitrate and Fe(III)-reduction while in the reduced horizons sulfate-reduction occurred. The higher variability between triplicate samples from the oxidized horizons might result from the small scale heterogeneity of these horizons. BElc and Btlc are grey-red mottled indicating the presence of small scale niches with different redox conditions, which would accommodate a greater variety of microorganisms, while Br1 and Br2 are evenly colored.

Quantification of *Geobacteraceae*, a family of well-known Fe(III)-reducers, in the different soil horizons yielded about 10^6 *Geobacteraceae* genomes per gram soil (ww). Highest genome copy numbers were detected in the oxidized horizon BElc, where conditions were appropriate for nitrate reduction, whereas, in Btlc, where conditions were appropriate for Fe(III)-reduction, significantly less copy numbers were detected. Compared with total cell counts obtained previously by DAPI-staining and -counting *Geobacteraceae* genome copies made up 0.2 – 0.5% of the total community in BElc soil but only 0.02 – 0.04% in Btlc soil. This could indicate the sensitivity of *Geobacteraceae* towards metal stress, as pore water and solid phase metal concentrations were enhanced in this soil horizon (Burkhardt et al., 2009). *Geobacteraceae* copy numbers were low compared with those obtained by Taq-Man-PCR by

Holmes et al. (2002) for sediments without in-situ Fe(III)-reduction (0.002 – 0.02 ng vs. 1 - 10 ng *Geobacteraceae* 16S rRNA gene per gram soil or sediment, respectively). Values were also low compared to qPCR-data by Wan et al. (2005) from a column experiment (10^8 *Geobacteraceae* 16S rRNA gene sequences per g sediment). Other results, obtained by MPN-PCR by Snoeyenbos-West et al. (2000), Holmes et al. (2002), Petrie et al. (2003), and North et al. (2004), were not comparable with qPCR data, as MPN-PCR generally seemed to yield lower copy numbers.

MPN-analysis demonstrated the sensitivity of indigenous Fe(III)-reducing microorganisms to metals. However, pore water metal concentrations were much lower and might be tolerated. The metal concentrations tested reflect those which might be released from the soil solid phase by intense solubilization processes, suggesting that *in situ* metal concentrations could potentially inhibit Fe(III)-reduction. The number of culturable Fe(III)-reducers was low compared to total cell numbers observed in the soil. However, abundance of Fe(III)-reducers might be underestimated due to exclusion of some organisms by cultivation conditions during MPN-analysis.

Metal tolerance of indigenous Fe(III)-reducers. Tolerance of Fe(III)-reducing microorganisms was generally low compared to values published for *Cupriavidus metallidurans*, *Escherichia coli*, *Pseudomonas fluorescens*, and sulfate-reducing microorganisms, which tolerate mM concentrations of several metals (Nies, 2003, Workentine et al., 2008, Sitte et al., in press). However, pore water metal concentrations were tolerated by the Fe(III)-reducing enrichment cultures obtained from Btlc-soil. Metal concentrations of the bioavailable fraction of the soil solid phase were toxic for Fe(III)-reducers. This was mainly due to Cu and Ni, and partially Zn.

Metal tolerance of Fe(III)-reducers indigenous to the Btlc-horizon was in the µmolar range, comparable to tolerances of *Shewanella* species which have been reported in former studies (Toes et al., 2008, Stone et al., 2006). In these studies growth of different *Shewanella* strains was completely inhibited at 150 µM Co, 150 - 400 µM Zn, 75 - 150 µM Cd, and 150 - 400 µM Cu when cultivated aerobically in 10% LB-broth (Toes et al., 2008). Hematite bioreduction by *S. putrefaciens* was 50% inhibited by 200 µM Zn under nongrowth conditions (Stone et al., 2006). The nutrient load of the medium influenced the tociticy of Cu and in more nutrient rich media higher tolerances of *Shewanella* were observed. Depending on nutrient load Cu tolerance was between 75 and 750 µM (Toes et al., 2008). The presence

of manganese oxides reduced the toxicity of Cu supposedly by decreasing its bioavailability due to sorption processes (Toes et al., 2008). It was suggested that Cu released during manganese oxide reduction re-precipitates to the solid phase as long as manganese oxides are present. After depletion of manganese oxides toxicity effects could be observed. Cultures grown on hydrous ferric oxides were more susceptible to Cu (Toes et al., 2008). In contrast to these low metal tolerances no inhibition of hematite reduction could be observed up to 1.63 mM Ni in a study by Paul and Stone (2009) where *S. putrefaciens* was incubated under non-growth conditions. For *Clostridium*, which can use reduce Fe(III) as a sink for excess electrons from fermentation, 24 µM Cd did not effect growth, but 24 µM Cu immediately inhibited growth (Francis and Dodge, 1988). However, the bioavailability of metals in cultures is decreased, due to interaction with media components. Only 49%, 5%, up to 72% or 14% of Ni, Zn, Cu, or Cd, respectively, were dissolved in the medium of non-inoculated controls and even lower amounts, 23%, 1%, up to 2%, or 2%, respectively, were dissolved in the inoculated enrichment cultures. In most previous studies dissolved metal concentrations were not determined, and therefore metal tolerances might be even lower than previously reported. As described above, the effect of the medium on metal toxicity was pointed out in a study by Toes et al. (2008). These data demonstrate the importance of determination of dissolved and bioavailable metal concentrations. However, considering dissolved metal concentrations, similarly low metal tolerance was observed in Fe(III)-reducing communities in soils. Solution concentrations of 2.8 – 1503 µM Cd inhibited 50% of Fe(III)-reduction in different soils (Welp and Brümmer, 1997). The toxic concentration of Cd in the soil was dependent on the soil type (organic matter content, sorption abilities, pH).

Identification of metal tolerant microorganisms. In a mixed clone library of Cu, Ni, and Zn amended cultures *δ-Proteobacteria* and *Firmicutes* were important phylogenetic groups, which is similar to clone libraries from biostimulated soil microcosm studies published previously (Burkhardt et al., 2010). All *δ-Proteobacteria*-clones were related to *Geobacter*, a genus well known for Fe(III)- and U(VI)-reduction (Lovley et al., 1991, Gorby and Lovley, 1992, Wilkins et al., 2006), and dominant during uranium reduction in bioremediation experiments (Holmes et al, 2002, Anderson et al., 2003). Within the *Firmicutes* several genera were detected. These organisms are known to reduce iron by shunting electrons from fermentation to Fe(III) (Francis and Dodge, 1988). In a previous study (Burkhardt et al., 2010) also *Acidobacteria* and *β-Proteobacteria* were important groups. *Acidobacteria* were not

detected in this study, which might be due to a higher metal sensitivity or they could not compete in the presence of a mixed carbon source (ethanol and lactate). Only one *β-Proteobacteria* clone was observed in this study, which was related to *Dechloromonas*. This genus reduces nitrate, sulfate, and apparently Cr(VI) and Se(VI) (Horn et al., 2005, Chung et al., 2006) and has been observed before in uranium-contaminated soil microcosms (Akob et al., 2008, Mohanty et al, 2008). However, the clone library contains the 1.1 µM Cu treatment, which did not show inhibition of Fe(III)-reduction. Therefore, the clone library might include also less metal tolerant organisms.

In metal tolerant Fe(III)-reducing enrichment cultures only few bands were detected by DGGE indicating a dominance of few organisms in metal tolerant Fe(III)-reducing microbial communities. Diversity might be limited due to exclusion of metal sensitive microorganisms, in addition to the enrichment of certain microbial groups by culture conditions. In a previous study, metal pollution reduced the bacterial diversity in soil by more than 99.9% and especially rare taxa disappeared (Gans et al., 2005) and the composition of the culturable bacterial community of a lake sediment was altered in the presence of 59 mg Pb kg^{-1} soil (Grandlic et al., 2006).

In contrast to the clone library, sequencing of the dominant DGGE bands of the Zn amended culture, which were also present in a 150µM Ni and 1.1µM Cu treatment, indicated a clear dominance of *Firmicutes* in the metal tolerant communities, although we can not be sure that bands in the same position are caused by the same organisms in all cultures. In the Ni and Cu amended cultures more bands, which could not be identified, were detected than in the Zn amended cultures. The *Firmicutes* detected in the Zn amended culture were related to fermenters of the genus *Sedimentibacter* and the order *Clostridiales*. *Sedimentibacter* strains have been found frequently in dechlorinating enrichments (Breitenstein et al. 2002, Cheng et al. 2010) and were necessary for β-hexachlorocyclohexane-dechlorination in a co-culture with *Dehalobacter*, probably due to excretion of growth factors (van Doesburg et al. 2005). This genus was also detected in a tar oil-impacted aquifer (Winderl et al. 2008). The closest cultured relative of the *Sedimentibacter*-clone in our study was *Sedimentibacter saalensis* (94% sequence identity), which was isolated form a dechlorinating enrichment originating from freshwater sediment (Breitenstein et al. 2002). The *Clostridiales* related clone shared highest 16S rRNA gene sequence identity with a clone obtained from a tar oil-impacted aquifer (Winderl et al. 2008). The closest cultured relative was *Clostridium aminobutyricum* with 95% sequence identity. In a former study the importance of a fermentative *Clostridium*

for microbial reduction of Cu-contaminated ferric oxide was demonstrated (Markwiese and Colberg, 2000). Although the fermenter reduced only a minor amount of the iron, they seemed to ameliorate metal toxicity towards Fe(III)-reducers by binding dissolved Cu. Fe(III)-reduction only started after dissolved Cu concentrations decreased to 5 ppb.

An isolate, DA-1, originating from Cd or Cu amended enrichments was closely related to *Desulfosporosinus lacus*. This genus has also been detected in a previous study in Btlc-microcosms (Burkhardt et al. 2010), where it seemed to be involved in sulfate-reduction. However, no sulfate was added to the enrichment cultures. *D. lacus* has been described to reduce sulfate, thiosulfate, sulfite, and, unlike other strains, also Fe(III) in the presence of lactate and can grow fermentatively with pyruvate and lactate (Ramamoorthy et al. 2006). Although *D. lacus* was isolated from a pristine environment, members of this genus have regularly been detected in various contaminated sites, including radionuclide contaminated sediment (Suzuki et al. 2002, Shelobolina et al. 2003, Nevin et al. 2003). *D. lacus* tolerated 2 mM As, but was inhibited by 10 mM As, 10 µM Cd, 0.4 mM Cr, and 10 µM Zn (Ramamoorthy et al. 2006). These concentrations are similar to dissolved metal concentrations in the enrichments were DA-1 was isolated from (23 µM Cd or 14 µM Cu). Altogether, these data suggested, that DA-1 probably reduced Fe(III) in enrichment cultures.

Conclusions. Our results indicate that metal tolerance of Fe(III)-reducers might be low in general. Thus, Fe(III)-reduction in the presence of metals, even at low concentrations, might be dependent on a metal scavenging process or on the presence of a sufficient carbon source to fuel detoxification. However, only little is known about the impact of metal contaminants on microbial Fe(III)-reduction and associated processes in soils, like metal retention, and more research is needed. Additionally, our results provide evidence for the importance of *Firmicutes* for microbial Fe(III)-reduction, especially in the presence of metal contaminants.

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Figure legends

Figure 1: Nitrate- and Fe(II)-concentrations of the pore water in the soil profile at the bank of the contaminated creek Gessenbach as measured in six or fife monthly measurements in 2007, respectively. The corresponding soil horizons are given at the right side of the graphs. This figure includes data published by Burkhardt et al. (2010) and Sitte et al. (in press).

Figure 2: Concentrations of selected metals in fractions obtained by sequential extraction from horizon Btlc.

Figure 3: Microbial community DGGE patterns generated from triplicate samples of each soil horizon and grouping of these patterns by cluster analysis.

Figure 4: Initial, non-biostimulated Fe(II)-formation rates obtained in soil-microcosms of horizons Btlc, Br1, and Br2, and biostimulated rates obtained in carbon-amended Btlc-microcosms in relation to the initial Fe(II)-concentration. This figure includes data published by Burkhardt et al. (2010) and Sitte et al. (in press).

Figure 5: Microbial community DGGE patterns generated from metal amended cultures and triplicate Btlc soil samples. The main bands that were identified by sequencing are labelled and the top Blast hit is given with its accession number and percentage of identity.

Figure 1

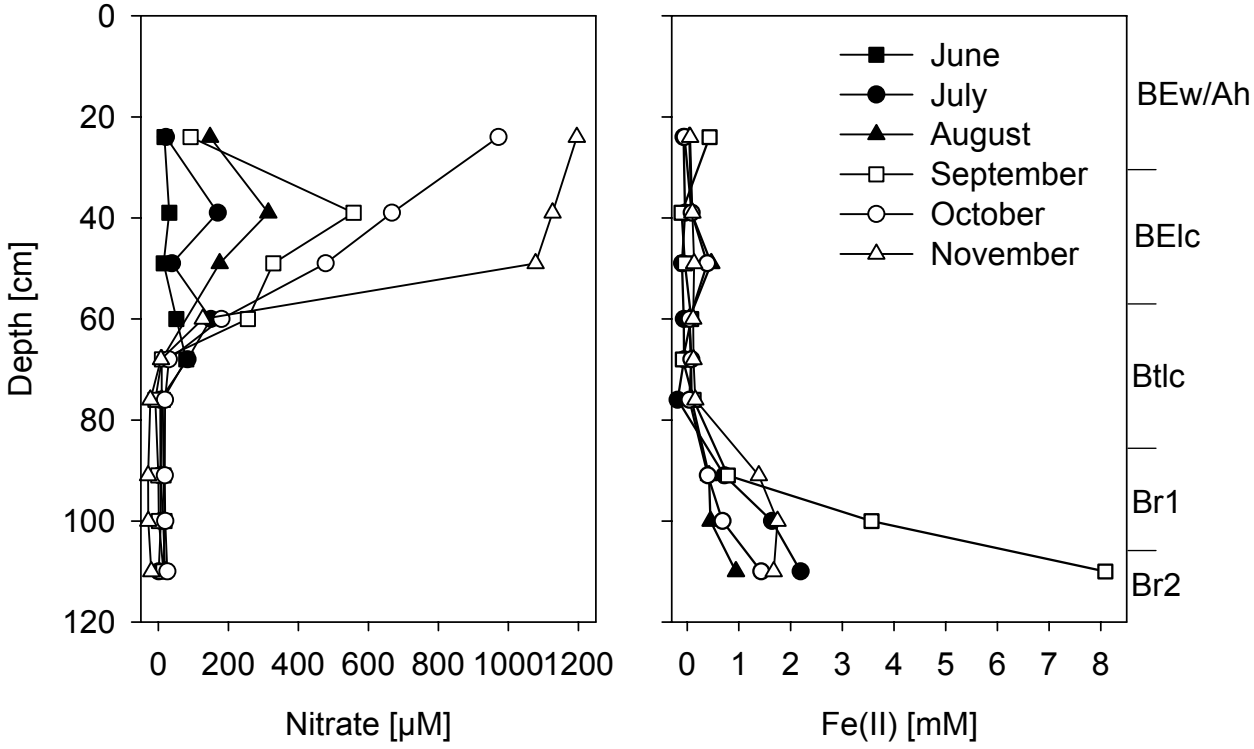

Depth [cm]
0
20
40
60
80
100
120
0 200 400 600 800 10001200
Nitrate [µM]
0 1 2 3 4 5 6 7 8
Fe(II) [mM]
June
July
August
September
October
November
BEw/Ah
BElc
Btlc
Br1
Br2

Figure 2

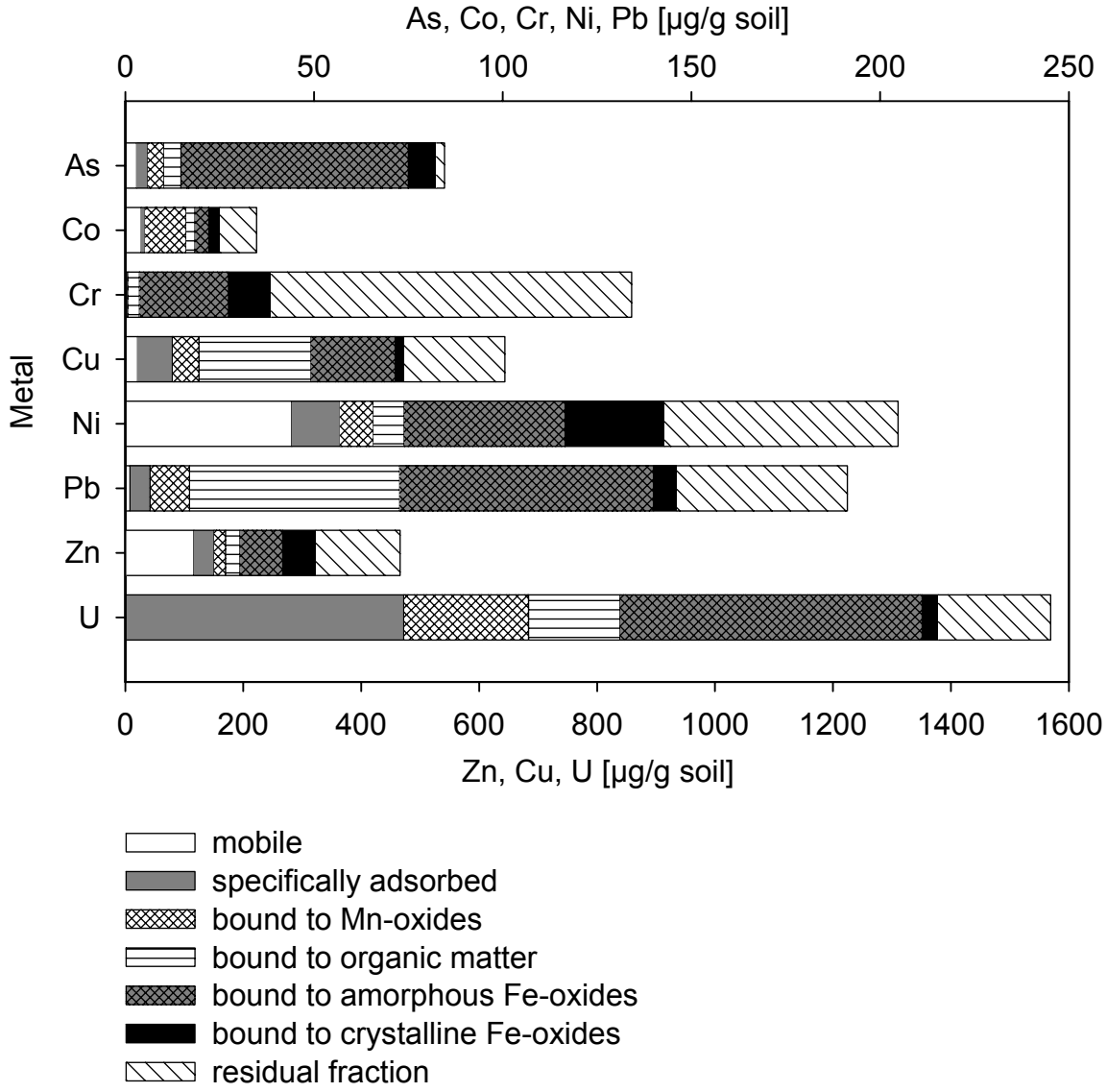

As, Co, Cr, Ni, Pb [µg/g soil]
0
50
100
150
200
250
Metal
As
Co
Cr
Cu
Ni
Pb
Zn
U
0
200
400
600
800
1000
1200
1400
1600
Zn, Cu, U [µg/g soil]
mobile
specifically adsorbed
bound to Mn-oxides
bound to organic matter
bound to amorphous Fe-oxides
bound to crystalline Fe-oxides
residual fraction

Figure 3

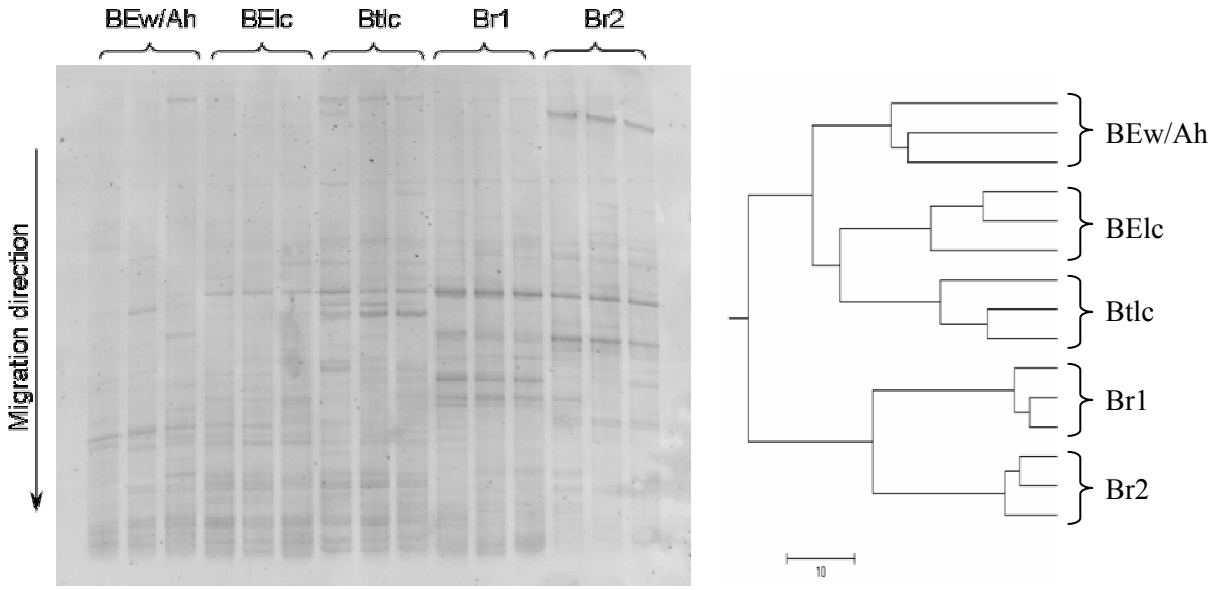

BEw/Ah
BElc
Btlc
Br1
Br2
Migration direction
BEw/Ah
BElc
Btlc
Br1
Br2
10

Figure 4

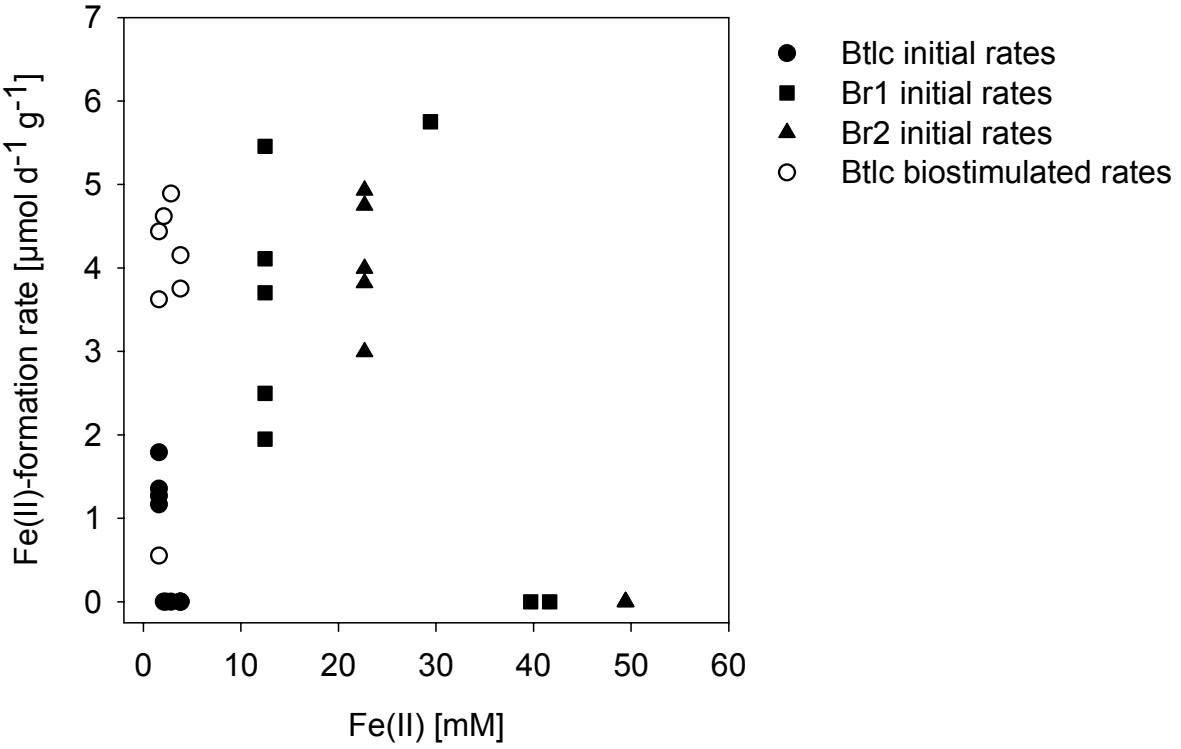
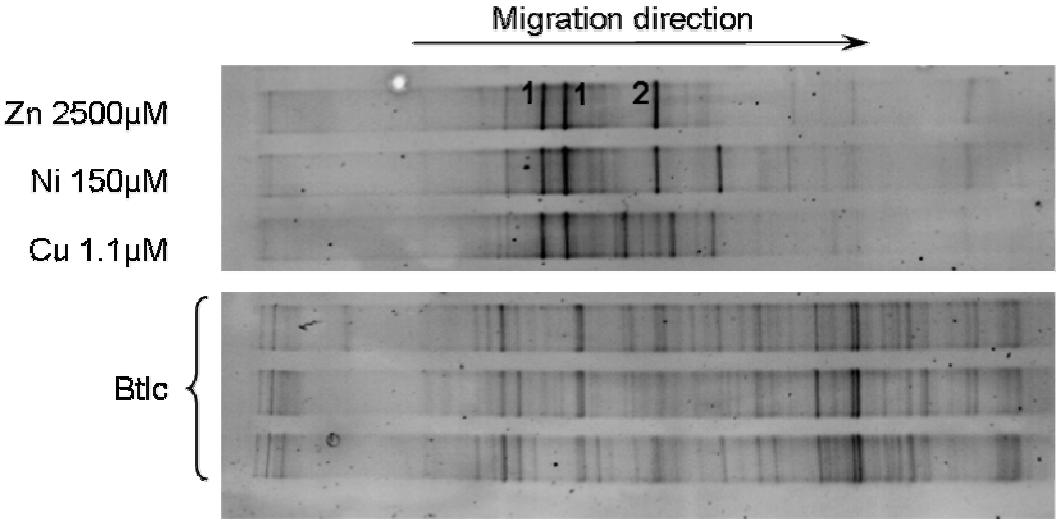

Fe(II)-formation rate [µmol d-1 g-1]
Fe(II) [mM]
0
1
2
3
4
5
6
7
0
10
20
30
40
50
60
Btlc initial rates
Br1 initial rates
Br2 initial rates
Btlc biostimulated rates

Figure 5



1 Sedimentibacter sp. clone VE117 (EF681724.1), 99% identity

2 Clostridiales bacterium clone D10_24 (EU266794.1), 99% identity

Table 1: Ct-values obtained by *Geobacteraceae*-specific quantitative PCR on 10 ng genomic DNA from soil samples and pure cultures. Each value was obtained by triplicate measurements of the same DNA sample. For soil DNA samples average values and standard deviations from triplicate soil samples are given, except for Br1 were duplicate soil samples were used.

Sample	Ct-value ± standard deviation
BElc	22.10 ± 0.46
Btlc	23.30 ± 0.36
Br1	21.88 ± 0.11
Br2	22.08 ± 0.30
Geobacter bremensis (DSM 12179)	12.39
Geobacter FRC-32	26.85
Geobacter metallireducens (DSM 7210)	23.58
Geobacter pelophilus (DSM 12255)	22.5
Geobacter sulfurreducens (DSM 12127)	14.3
Desulfuromusa bakii (DSM 7345)	33.83
Acidiphilium cryptum (DSM 2389)	29.54
Acetobacterium woodii (DSM 1030)	24.91
Bacillus subtilis subsp. *subtilis* (DSM 402)	29.22
Clostridium glycolicum (DSM 1288)	33.77
Sporomusa rhizae (DSM 16652)	33.24
Non-template control	36.17

Table 2: Maximum pore water metal concentrations in horizon Btlc.

Metal	Maximum concentration [µM]
Al	3.85
As	0.06
Cd	0.27
Co	0.63
Cr	0.01
Cu	1.52
Ni	16.43
Pb	0.01
U	0.08
Zn	38.56

Table 3: Genome copy numbers per gram fresh weight soil obtained by *Geobacteraceae*-specific quantitative PCR on genomic DNA from soil samples. Average values and standard deviations from triplicate soil samples are given, except for Br1 were duplicate soil samples were used. For each DNA sample triplicate measurements were perfomed.

Soil sample	Genome copies[a] g soil (ww)$^{-1}$ ± standard deviation
BElc	$5.86 * 10^6 \pm 2.18 * 10^6$ [A]
Btlc	$1.44 * 10^6 \pm 0.59 * 10^6$ [B]
Br1	$0.53 * 10^6 \pm 0.07 * 10^6$ [B]
Br2	$0.82 * 10^6 \pm 0.35 * 10^6$ [B]

[a] Genome copies are calculated based on 16S rRNA gene copy number of *Geobacter sulfurreducens*.
[A, B] Groups of soil horizons with significant different genome copy numbers g soil (ww)$^{-1}$

Table 4: MPN estimates of Fe(III)-reducing microorganisms from Btlc-soil cultured at pH 5.2 with two different carbon sources in the presence or absence of supplemented heavy metals. Goethite was used as an electron acceptor. The values were estimated from 3 replicates.

Metabolic type	MPN g (ww soil)$^{-1}$ (95 % confidence limit)	
	Without heavy metals	With heavy metals
Lactate-utilizing Fe(III)-reducers	$9 * 10^4$ ($1.9 * 10^4 – 4.2 * 10^5$)^A	$0.4 * 10^1$ ($0.9 * 10^0 – 1.8 * 10^1$)^B
Glucose-utilizing Fe(III)-reducers	$1.5 * 10^4$ ($3.2 * 10^3 – 7.0 * 10^4$)^A	$2.3 * 10^1$ ($0.5 * 10^1 – 1.1 * 10^2$)^B

$^{A, B}$ Groups with significant differences of the MPN values. Significance is given when the ratio between MPNs is above 8.87 (Alef, 1991).

Table 5: Fe(II)-formation rates in Fe(III)-reducing cultures grown in the presence of metals.

Treatment	Concentration added [µM]	Elongated lag phase?	Intensity of Fe(II)-formation[a]
Control	No metal added	No	++
Mix 1	See foot note [b]	No	++
Mix 2	See foot note [c]	Yes	-
$CdCl_2$	0.15	No	++
	0.3	No	++
	15	No	++
	30	No	+
$CoCl_2$	0.15	No	++
	0.35	No	++
	50	No	++
	150	Yes	++
$CuCl_2$	0.5	No	++
	1.1	No	++
	150	Yes	-
	1500	Yes	-
$NiCl_2$	10	No	++
	20	No	++
	150	No	+
	1000	Yes	-
$CrCl_3$	0.06	No	++
	1	No	++
	10	No	++
	100	No	++
$ZnCl_2$	20	No	++
	40	No	++
	500	No	++
	2500	Yes	+
$AlCl_3$	2	No	++
	4	No	++
	100	No	++
	1500	No	++
$PbCl_2$	3	No	++
	6	No	++
	20	No	++
	100	No	++

[a] ++/+/- Fe(II)-formation rates: 300-560, 50-300, 0-50 [nMol FeII d^{-1} mL^{-1}], respectively.
[b] Amendments for mix 1: 0.3µM $CdCl_2$, 0.35µM $CoCl_2$, 1.1µM $CuCl_2$, 20µM $NiCl_2$, 0.06µM $CrCl_3$, 40µM $ZnCl_2$, 4µM $AlCl_3$, 6µM $PbCl_2$
[c] Amendments for mix 2: 30µM $CdCl_2$, 150µM $CoCl_2$, 1500µM $CuCl_2$, 1000µM $NiCl_2$, 10µM $CrCl_3$, 2500µM $ZnCl_2$, 1500µM $AlCl_3$, 20µM $PbCl_2$

Table 6: Summary of phylogenetic affiliation and distribution of 16S rRNA gene clones from clone libraries of metal amended enrichment cultures.

Phylogenetic group	Clone designation	Nearest relative (accession number)	% Identity	Number of clones in phylotype
Betaproteobacteria	SB60	*Dechloromonas* sp. MissR (AF170357)	99	1
Deltaproteobacteria	SB3	Clone IRD18B06 (AY947909)	97	8
	SB8	*Geobacter psychrophilus* (AY653549)	98	21
	SB54	*Geobacter* sp. Ply1 (EF527233)	98	2
Firmicutes	SB4	*Anaerobranca* sp. Clone SRB2 (DQ069229)	96	3
	SB5	*Sedimentibacter* sp. Clone JN18_A14_H (DQ168650)	99	2
	SB18	*Sedimentibacter* sp. Clone BRS2 (AY221992)	98	4
	SB14	Clone BSV54 (AJ229206)	98	5
	SB25	Clone BSV34 (AJ229194)	97	2
	SB52	*Clostridiaceae* clone dgC-140 (AB218342)	92	1
	SB15	Clone ZZ14C3 (AY214191)	98	1

The influence of intracellular storage material on bacterial identification by means of Raman spectroscopy

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Abstract

Micro-Raman spectroscopy is a suitable method for single cell microbial identification due to its non-invasive character and minimal or no sample preparation requirement. Raman spectra yield fingerprint-like information about all chemical components within one cell and, combined with multivariate methods, differentiation down to species or even strain level is possible. Many microorganisms may accumulate high amounts of polyhydroxyalkanoates (PHA) as carbon and energy storage materials within the cell and the Raman bands of PHA might impede the identification and differentiation of cells. To date, the identification by means of Raman spectroscopy have never been tested on bacteria which had accumulated PHA. This study aims to identify different bacterial species capable of intracellular polymer accumulation as a storage material and to differentiate between cells growing under different conditions. Combining fluorescence imaging and Raman spectroscopy, we identified polyhydroxybutyrate (PHB) as storage polymer accumulating in the cells. The amount of polymer energy storage material present was dependent on the physiological status of the microorganisms and strongly influenced the identification results. Bacteria in the stationary phase formed granules of crystalline PHB, which obstructed the Raman spectroscopy identification of bacterial species. The Raman spectra of the bacteria in the exponential phase were dominated by signals from the storage material. However, the bands from proteins, lipids, and nucleic acids were not completely obscured by signals from PHB. Cells growing under either oxic or anoxic conditions could also be differentiated, suggesting that changes in Raman spectra can be interpreted as an indicator of different metabolic pathways. Although the presence of PHB induced severe changes in the Raman spectra, our results suggest that Raman spectroscopy can be successfully used for identification as long as the bacteria are not in the stationary phase.

Introduction

An important concern in microbiology is to find a rapid method for reliable microbial identification. Ultimately, the goal in microbial identification is to link microbial cell identification with metabolic potentials *in situ*, i.e. on a single cell level [1]. Microbial ecology studies the interactions of microorganisms with their biotic and abiotic environment. Since these interactions determinate the occurrence, distribution and activities of microbial species, to be able to describe an interaction, information about microbial species present in the environment and their activity are required [2].

Culture-dependent approaches are limited, because only a minority of microorganisms can be cultivated with standard techniques in the laboratory that do not mimic *in situ* conditions [3]. The high cost and complexity of the molecular methods developed over the last two decades and currently used for identification make them unsuitable for routine analyses [4].

Alternatively, spectroscopic methods can be used for single cell identification. Recently, spectroscopic methods based on the physicochemical property of whole cells have been used to identify microorganisms [5-11]. Compared with other spectroscopic techniques, the advantages of this micro-Raman spectroscopy technique are its non-invasive character, minimal or no sample preparation, and the fact that only one bacterial cell is required to record the fingerprint Raman spectrum which is used for microbial identification [12-14]. Variations in the biochemical composition between different microbial species enable their identification by means of Raman spectroscopy [5, 12, 15]. Raman spectroscopy is used not only for bacteria analyses, but also for studies focused on plants and human cells [16-21]. In general, Raman spectroscopy has the potential for broad applicability, as it is successfully used in chemistry, material sciences, mineralogy, art, and archeology [22-24].

Raman spectra yield fingerprint-like information about all chemical components within the cell. Information about the functions of microorganisms in complex bacteria communities can also be collected using Raman spectroscopy alone [25] or in combination with other methods, e. g. FISH [10]. Identification to the strain level is also possible with Raman spectroscopy [26-32]. To perform Raman spectroscopic identification, an algorithm to differentiate between different microorganisms' taxa is required and various statistical methods are applied to analyze the Raman signals. For a large Raman database, a dimensionality reduction of the data can be performed using principal component analysis (PCA), prior to any spectra classification. Classification or identification in previous studies was accomplished by either an unsupervised method (hierarchical cluster analysis (HCA)) or

a supervised one (K-nearest neighbor classifier (KNN), nearest mean classifier (NMC), linear discriminant analysis (LDA), or support vector machines (SVM)) [8, 26, 30, 31, 33]. Support vector machines have a wide variety of biological applications, including the classification of proteins and DNA sequences, microarray gene expression profiles [34, 35] or microorganisms [8, 11].

In order to make the transition from poof-of-principle studies to studies under field conditions, the influence of various parameters must to be investigated. For example, many microorganisms produce polyhydroxyalkanoates (PHA) as carbon and energy storage materials, but also as an electron sink, usually under conditions of limited nutrients [36, 37]. Polyhydroxybutyrate (PHB) is the simplest and the most common PHA and has strong potential as a biodegradable polymer [38-40]. Consequently, numerous studies have been dedicated to increasing the rate of polymer accumulation in cells [36, 41-43], detection [44], and quantification [45, 46] of the quantity of PHB stored by microorganisms. High amounts of these polymers have been detected in some bacteria, with concentrations of PHB up to 80% of the total dry weight reported [47]. Thus, the Raman bands generated by polyhydroxybutyrate might impede the identification and differentiation of some bacterial cells. However, none of the microorganisms identified by means of Raman spectroscopy up to this point have contained storage materials.

An identification method based on single cell analysis is valuable in e.g. microbial ecology since traditional cultivation-based methods are slow and, due to their inevitable selectivity, usually fail to achieve a complete picture of the true composition of complex communities [3]. A relatively large number of microorganisms are able to produce and accumulate within the cells various energy storage polymers [37], therefore a study focused on the effects of PHB on microbial identification using Raman spectroscopy is required. Furthermore, identification of new strains which accumulate high amount of storage materials could positively influence the field of biodegradable materials since these microbes could be used for the production of polymers.

In this paper we evaluate the robustness of the Raman spectroscopic approach in combination with statistical methods, for the identification of microorganisms. We investigate the effect of PHB accumulation in bacterial cells belonging to different classes on the identification results obtained by combining Raman spectroscopy with SVM.

Experimental procedures

Sample preparation. The microorganisms *Bacillus megaterium* DSM 90 (Firmicutes; Gram-positive), three strains of *Bacillus thuringiensis* (DSM 530, DSM 5725 and ATCC10782) (Firmicutes; Gram-positive), *Azohydromonas lata* DSM 1122 (Betaproteobacteria; Gram-negative) and *Cupriavidus necator* DSM 428 (Betaproteobacteria; Gram-negative) were cultivated on NA (nutrient agar) medium at 30°C. *Acidiphilium cryptum* JF-5 (Alphaproteobacteria; Gram-negative) was cultivated either in liquid TSB (tryptone soya broth) medium enriched with 2mM glucose at 32°C under oxic conditions or under anoxic conditions with amended soluble $Fe_2(SO_4)_3$ (30mM) as electron acceptor. From each liquid sample, an amount of 1.5 ml of culture was centrifuged 3 times at 12000g for 2 minutes and washed in each case with distilled water. In case of bacteria cultivated on agar plates, a part of the colony was scraped from plates using an inoculating loop, suspended in 1.5 ml of water and washed as above mentioned. Thereupon a droplet of suspension was dried on air at room temperature on a fused silica surface. For all analyzed bacterial strains, the Raman spectra from at least two independent cultures obtained from different sets of media were recorded.

Fluorescence staining and observation. A solution with the concentration of 1g/l was prepared by dissolving Nile red with dymethyl sulfoxide (DMSO). A 1 ml aliquot of the culture was mixed with 0.3 µl of Nile red solution and incubated for 30 minutes. After incubation, the culture was centrifuged 3 times at 12000g for 5 minutes and washed in each case with distilled water. Afterwards, a droplet of suspension was dried on air at room temperature on a fused silica surface. Subsequently, fluorescence imaging observations were performed. The fluorescence imaging observations were made by means of an inverse microscope (BX 41, Olympus) coupled to a CCD camera (CC12, Olympus). The fluorescence excitation is realized with a mercury lamp, different excitation and emissions wavelengths were obtained by exchanging the filters in the light path.

Spectroscopic instrumentation. The Raman spectra of the bacteria were recorded with a micro-Raman setup (HR LabRam inverse system, Jobin Yvon Horiba). Raman scattering was excited by a frequency doubled Nd:YAG laser at a wavelength of 532 nm with a laser power of about 2 mW incident on the sample. The laser beam was focused on individual cells by means of a Leica PLFluoar x100/0.75 microscope objective down to a spot diameter of approximately 0.7 µm, which is sufficient to resolve a single bacterium from the background.

The dispersive spectrometer has an entrance slit of 100 µm, a focal length of 800 mm and is equipped with a grating of 300 lines mm^{-1}. The Raman scattered light was detected by a CCD camera operating at 220 K. The acquisition time per spectrum was 60 s. The data were acquired over the course of several days. For the calibration procedure, titanium dioxide (anatase) was measured daily as a reference control.

Chemometric analysis. The analysis of Raman spectra was performed in two steps: (i) preprocessing of the spectra and (ii) identification by using SVMs. A support vector machine is a computer algorithm that learns by example to assign labels to objects [35]. The SVM always solves two-class problems, therefore a hyperplane is defined in order to discriminate the two classes. The hyperplane is characterized by the support vectors which are data points belonging to one class and having the highest similarity to the other class [8].

The preprocessing was tested by different methods such as baseline correction, normalization, first and second derivative. Baseline correction using the SNIP algorithm [52] plus vector normalization for single-bacterium analysis are the best obtained results and therefore were used for further preprocessing of the data. Baseline correction was applied to remove the background generated by the fluorescence emission. The SNIP baseline estimation was used because of the speed and the reliability of this method. To avoid the variation in the Raman intensity generated by the difference in the mass of the microbial cells, the vector normalized finger print region was used in all further computation. Thereafter, a PCA [53] was performed, the first 20 scores being used for further data analysis. The dimension reduction was done first to reduce the cost of the computation, second to remove white noise from the spectrum and third to avoid overfitting. With this data set of scores a one-against-one [54] system of support vector machines [55] with a radial basis kernel was trained and tested on a completely independent set. With a one-against-the-rest schema it is possible to build up a multiclass classifier out of single binary classifiers. The training set was composed from 266 Raman spectra recorded from five species. For the identification 102 independent Raman spectra were used.

Results and discussion

The Raman spectrum of a microorganism is the sum of Raman signals of the individual cell components. Therefore, contributions from proteins, nucleic acids, and lipids are expressed in the spectrum. A typical bacterial spectrum (Figure 1 – spectrum a) shows two bands

correlated to the presence of proteins in the microorganism: two bands at approx. 1660 cm^{-1} and 1242 cm^{-1} are assigned to amide I and amide III vibrations, respectively. Contributions from the amino acids are detected at 1001 cm^{-1}, a sharp band characteristic of phenylalanine, and approx. 1610 cm^{-1}, which is assigned to phenylalanine and tyrosine. The band at 1575 cm^{-1} is specific to nucleic acid ring stretching vibration, especially of guanine and adenine, while the band at 1314 cm^{-1} is assigned to CH_2/CH_3 deformation vibrations [12, 32]. Another signal belonging to CH_2 deformation is the band at 1342 cm^{-1}, the band at 1450 cm^{-1} is given by the CH_2 scissoring deformation [12]. The most prominent feature within the Raman spectrum of bacteria is the broad band centered at 2932 cm^{-1} which is the superposition of the CH stretching vibrations of CH_2 and CH_3 groups in proteins, lipids, nucleic acids and carbohydrates [12, 32]. The broad bands at 785 and 1075 cm^{-1} labeled with asterisk in Figure 1, are generated by the fused silica slides on which the investigated bacteria were located.

The average spectra displayed in Figure 1, each spectrum being the average of 20 single cell spectra, were recorded from a culture of *Bacillus megaterium* DSM 90. The main difference between the two spectra is the signal at 1735 cm^{-1} in spectrum B which can be assigned to stretching vibration from a carbonyl bond. The appearance of this C=O signal is accompanied by a decrease in the intensity of protein and nucleic acid bands, while the intensities of CH deformation bands increase. The outcome seems at first surprising, since the Raman spectra of a single strain cultivated under various conditions are usually very similar, with chemometrical methods usually being required to differentiate between similar bacterial cells grown under different conditions [15]. However, the important differences between the two Raman spectra can be attributed to a new substance present in large quantities in the cells. The decrease in the Raman signals of the other cell components likely influences the Raman spectroscopy identification process, as the protein and nucleic acid bands are crucial for the discrimination between various bacteria. The same decrease in intensity of the protein and nucleic acid bands was also noticed in the Raman spectra of *Acidiphilium cryptum* JF-5 (not shown here). Therefore, the starting point of this study was the identification of the substance responsible for the aforementioned changes in the Raman spectra. The analysis of the influence of this unknown substance on bacterial identification is vital for further applications of this spectroscopic method for classification and identification of microorganisms.

Many bacteria produce polyhydroxyalkanoates (PHA) as carbon and energy storage molecules and can accumulate high amounts of PHA in the cells [38]. To demonstrate that PHA was responsible for our observed changes in Raman spectra, a combination of

fluorescence imaging and Raman spectroscopy technique was used. The bacteria were first stained with a fluorescent dye (Nile red) to illuminate the cells under florescence which contained substrate storage materials. The cells containing PHA were then located on the slide surface by means of fluorescence imaging. By comparing the fluorescence images with bright field pictures, distinctions between cells with and without storage granules was possible. Further, Raman spectroscopy was applied to analyze the chemical composition of different cells. Figure 2 illustrates stained *A. cryptum* JF-5 cells, observed in brightfield image (A) and in fluorescence illumination (B), respectively. Based on the fluorescent images, two cells were selected for Raman measurements, one with and one without storage materials and the spectra obtained are presented in Figure 2(C). The Raman spectrum of the fluorescent cell is dominated by bands assigned to CH stretching vibrations at 2935 cm^{-1}, CH deformation vibrations at 1450 cm^{-1} and C=O stretching vibrations at 1735 cm^{-1}. The broad bands at 785 and 1075 cm^{-1} are assigned to the fused silica substrate on which the investigated bacteria were placed. The intensity of the protein and nucleic acid bands is very low. The Raman spectrum of the cell without storage materials (b) is dominated by the signals assigned to amide I, amide III and nucleic acids. In addition, the band assigned to C=O stretching vibration is completely absent. This indicates that a polyhydroxyalkanoate substance is likely responsible for the presence of the 1735 cm^{-1} band.

Next, we identified the polymer produced by *A. cryptum* JF-5. Although a large variety of PHA storage granules may be synthesized by microorganisms, by far the most common substance is polyhydroxybutyrate. The Raman spectrum of crystalline PHB is displayed in Figure 3 (c). The CH stretching band, centered at 2932 cm^{-1}, is the dominant signal in the spectrum, with five clearly separated bands being observed in this region. The signals are assigned to CH_2 and CH_3 symmetric and asymmetric stretching vibrations. In the region of 1350-1450 cm^{-1}, bands from various CH deformation vibrations are present. The Raman signals at 1402 cm^{-1} and 1364 cm^{-1} are assigned to CH_3 symmetric deformation, and the band at 1450 cm^{-1} to CH_3 as well as CH_2 deformation vibrations. The stretching vibration of the C=O double bond is located at 1735 cm^{-1}. The single bond CC stretching vibration is responsible for the Raman signal at 840 cm^{-1} and CO stretching vibration for the band at 1048 cm^{-1} [15, 48]. An overview of the tentative assignment of the Raman signals obtained from bacterial cells is presented in Table1.

A comparison between the PHB crystalline spectrum (Figure 3 (c)) and a spectrum recorded from a cell in exponential growth (Figure 3 (a)) reveals important differences. The

bands in the region 1200-1300 cm^{-1} are observed only in the crystalline PHB spectrum, the intensity of the CH_3 symmetric deformation vibrations (1364 and 1402 cm^{-1}) are very low in the bacterial spectrum, and the signal at 1735 cm^{-1} assigned to C=O stretching vibration is much broader in the bacterial spectrum than in crystalline PHB. Furthermore, in the CH stretching vibration region, five bands are clearly distinguished in the crystalline spectrum, while in the bacterial spectrum the bands are broader and form a single large band. However, the spectrum of amorphous PHB (Figure 3 (b)) is very similar to the spectrum obtained from a cell in the exponential phase with storage materials, indicating that PHB is the storage material present in bacterial cells. This also suggests that bacteria in exponential growth phase create deposits of polymers in the amorphous state. In case of bacteria in the stationary phase (Figure 4 (c)), the microorganism spectra are almost identical with the spectrum of crystalline PHB (Figure 3 (c)), suggesting that microorganisms in stationary growth phase accumulate storage materials in the crystalline form.

With increasing amounts of storage material in cells, Raman signals from other cell components decrease in intensity. This behavior likely affects identification since the protein and nucleic acid bands are used to differentiate between microorganisms. Therefore, we also investigated the influence of storage granules on bacterial identification. To select an appropriate time point for sampling, different growth phases of the culture were to be investigated, because the age of the cell can influence its chemical composition. Figure 4 shows differences between spectra of *A. cryptum* JF-5 obtained during different growing phases. The main differences between the spectra are due to different amounts of PHB stored in cells at each time point. Raman spectra of the cells in the lag phase showed no polymer accumulation (Figure 4 (a)). Starting from the exponential phase (Figure 4 (b)), PHB is stored within the cell, first as amorphous PHB, then, in the stationary phase (Figure 4 (c)), as crystalline. From this data, we determined that the appropriate time points for sampling were in the late exponential phase, when accumulation of storage material is still on-going, but the Raman signals of proteins and nucleic acids are not yet completely obscured by PHB. For microorganisms in the stationary phase, the Raman bands of crystalline PHB completely cover the signals from the other cell components, making the discrimination of the organisms in function of growing history impossible.

Raman spectra from various PHB producing Gram-positive or Gram-negative bacteria belonging to the Firmicutes or to different classes of the Proteobacteria were also recorded. We selected *A. cryptum* (Figure 5 (a)), *Bacillus megaterium* (Figure 5 (b)), *Azohydromonas*

lata (Figure 5 (c)), *Cupriavidus necator* (Figure 5 (d)) and *Bacillus thuringiensis* (Figure 5 (e)) for this study because of their tendency to accumulate PHB. All the spectra were very similar (Figure 5), requiring a chemometrical method to discriminate between the different species. Using support vector machine (SVM), a supervised identification method, a clear distinction between spectra belonging to various bacteria was possible, with a recognition rate of 90.1% (Table 2).The training set was formed by 266 spectra, belonging to the above mentioned species. Spectra from two different strains of *B. thuringiensis* (DSM 530 and ATCC 10782) were incorporated in the training dataset in the *B. thuringiensis* group. For the validation, spectra from bacteria cultivated in different batches than the microorganisms used for the training set where used. In addition, the Raman spectra were recorded at different days, to avoid possible errors in identification induced by measurement artifacts. To challenge the robustness of the identification model, an external dataset of spectra belonging to *B. thuringiensis* DSM 5725 was added to the validation dataset. From 102 spectra, 10 were misclassified: five *A. lata* spectra were assigned as *B. megaterium,* four *B.megaterium* spectra were assigned as *A. lata* and one *C. necator* spectrum again as *A. lata*. The spectra belonging to the *B. thuringiensis* DSM 5725 (strain which was not included in the training set) were all correctly assigned to the *B. thuringiensis* group. These results indicate that microbial identification of single bacterial cells by means of Raman spectroscopy can be performed, even if the cells produce storage materials.

We also investigated if bacterial cells of the same strain can be differentiated even if the cells grow under different conditions and some produce PHA. Raman spectroscopy can be used to discriminate bacterial cells which contain no storage granules, belong to the same strain but are grown in different environmental conditions [15, 49]. However, no information is available for bacteria which accumulate storage materials. For this investigation *A. cryptum* JF-5 was used, because it is facultative anaerobe and therefore can grow under both oxic and anoxic conditions, using oxygen (O_2) or ferric iron as an electron acceptor [50, 51]. In this experiment, *A. cryptum* JF-5 was incubated either with O_2 or with $Fe_2(SO_4)_3$ as an electron acceptor. The cells were harvested in the exponential phase, measured with Raman spectroscopy, and the spectra were analyzed using SVM. Performing a classification with 325 spectra, 307 spectra were correctly identified, which is a recognition rate of 94.5% (Table 3). Of the 18 spectra that were misclassified, eight spectra belonged to *A. cryptum* JF-5 cells grown anaerobically were assigned as spectra from cells grown aerobically. Ten spectra recorded from aerobically grown cells were assigned as spectra from anaerobically grown

cells. These data suggest that Raman spectroscopy can classify bacteria which use different metabolic processes for energy conservation even if the cells accumulate polymers as energy source. Because the cells either use O_2 or ferric iron as electron acceptor, the changes in the Raman spectra can be interpreted to determine the different metabolic pathways involved in *A. cryptum* JF-5 respiration. Therefore, Raman spectroscopy may be a useful tool to differentiate between cells even on the strain level, i.e. the lowest classification level, with different growth histories independent of the presence or absence of storage materials.

Conclusion. The production of storage materials depends on the physiological status of the microorganisms. Our study had to consider the age influence on the biochemical composition of the microbes and the subsequent effect on identification results. Bacteria accumulate storage materials in different forms depending on cell activity status. In stationary phase crystalline PHA is intracellularly stored while in exponential phase the polymer is present in amorphous state. Since identification of various bacteria belonging to different groups was determined only for bacteria which store amorphous PHA, our outcome shows the effect of the microorganisms' age on identification results. However, bacteria in the exponential phase were still successfully identified by means of micro-Raman spectroscopy, with even discrimination between bacteria belonging to the same species but grown under different conditions. To summarize, the present contribution demonstrates that the presence of polyhydroxybutyrate in microbial cells does not hamper Raman spectroscopy identification as long as the microorganisms are in the exponential growth pahse.

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Table and figure captions

Table 1 Tentative wavenumber assignment of some of the Raman signals of bacterial cells

Table 2 Identification of PHB producing bacteria via SVM.

Table 3 Classification of *A. cryptum* JF-5 in function of the utilized electron acceptor, by means of SVM.

Figure 1 Average Raman spectra of the *Bacillus megaterium* DSM 90 cells without (a) and with (b) PHB.

Figure 2 Microscope images of stained *A. cryptum* JF-5 cells with or without storage granules on a quartz substrate. The image (A) displays brightfield illumination and (B) is obtained from a fluorescence illumination at the same position like in (A). Raman measurements were performed on the cells marked with circles (C).

Figure 3 Raman spectra of: (a) spectrum single bacterium, (b) amorphous PHB and (c) crystalline PHB.

Figure 4 Raman spectra of *A. cryptum* JF-5 in different growth phase: (a) lag phase, (b) exponential phase, (c) stationary phase.

Figure 5 Raman spectra from various PHB producing bacteria: (a) *Acidiphilium cryptum*, *(*b) *Bacillus megaterium*, (c) *Azohydromonas lata*, (d) *Cupriavidus necator*, (e) *Bacillus thuringiensis*

Tables and figures

Table 1

Wavenumber (cm^{-1})	Tentative assignment
840	single bond CC stretching vibration
1001	phenylalanine
1048	CO stretching vibration
~1242	amide III
1314	CH_2/CH_3 deformation vibrations
1342	CH deformation
1365	CH_3 symmetric deformation
1402	CH_3 symmetric deformation
1450	CH_2 scissoring deformation
1575	nucleic acid ring stretching vibration, especially of guanine and adenine
1610	phenylalanine and tyrosine
~1660	amide I
1735	C=O stretching vibration

Table 2

	A. lata	*B. thuringiensis*	*C. necator*	*A. cryptum*	*B.* **megaterium**	*Correct %*
A. lata	**14**	-	-	-	5	*73.6*
B. thuringiensis	-	**20**	-	-	-	*100*
C. necator	1	-	**22**	-	-	*95.6*
A. cryptum	-	-	-	**20**	-	*100*
B. megaterium	4	-	-	-	**16**	*80*
Recognition rate						***90.1***

Table 3

	With $Fe_2(SO_4)_3$	**With O_2**	***Correct %***
With $Fe_2(SO_4)_3$	**137**	8	*94.5*
With O_2	10	**170**	*94.4*
			94.5

Figure 1

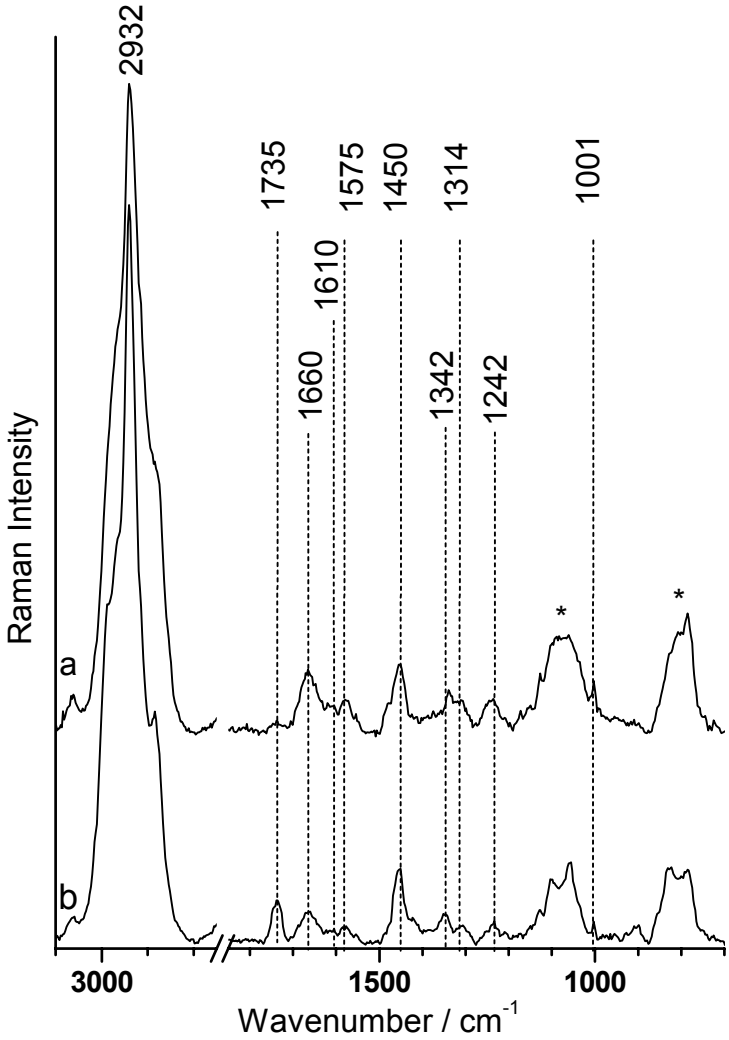

2932
1735
1575
1450
1314
1001
1610
1660
1342
1242
*
*
a
b
Raman Intensity
3000
1500
1000
Wavenumber / cm-1

Figure 2

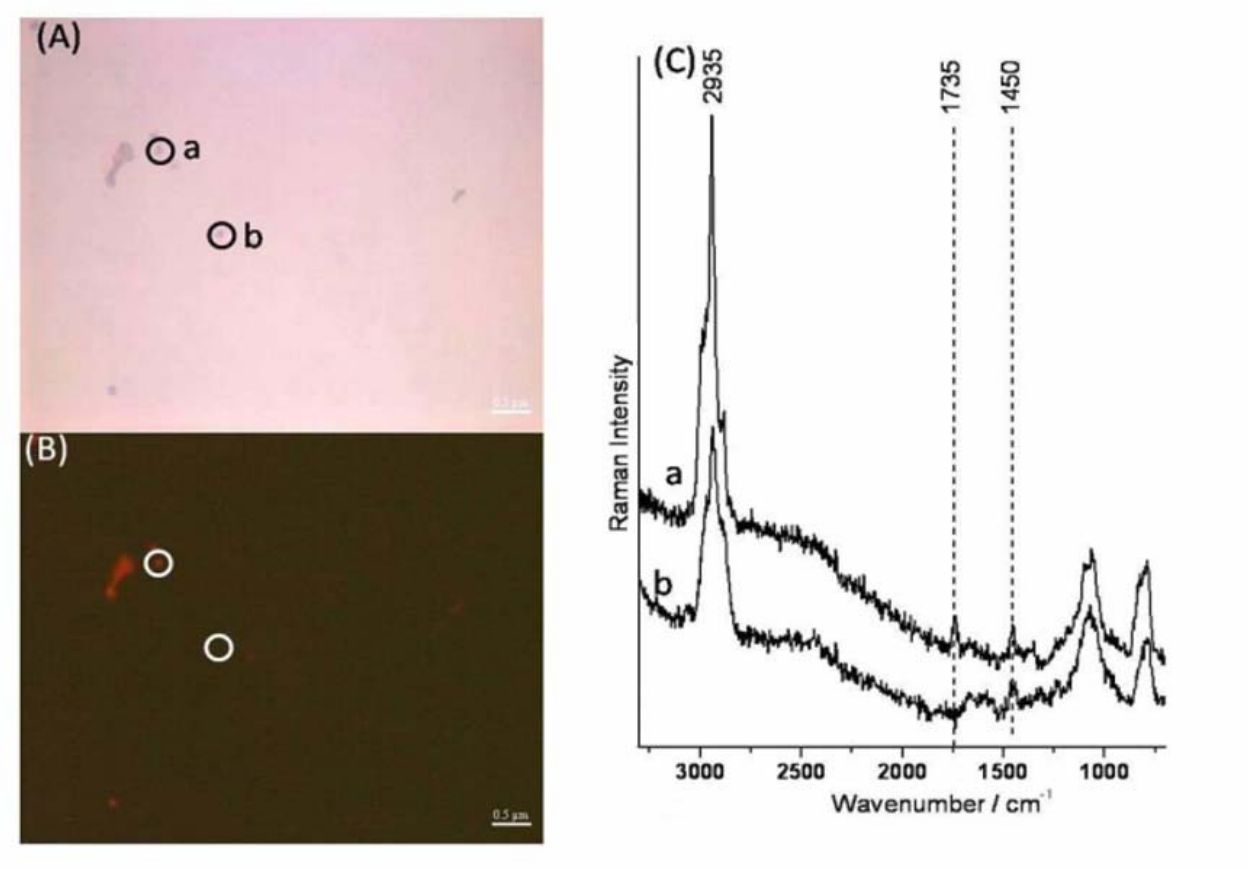

(A)
a
b
(B)
0.5 µm
(C)
2935
1735
1450
Raman Intensity
a
b
3000
2500
2000
1500
1000
Wavenumber / cm-1

Figure 3

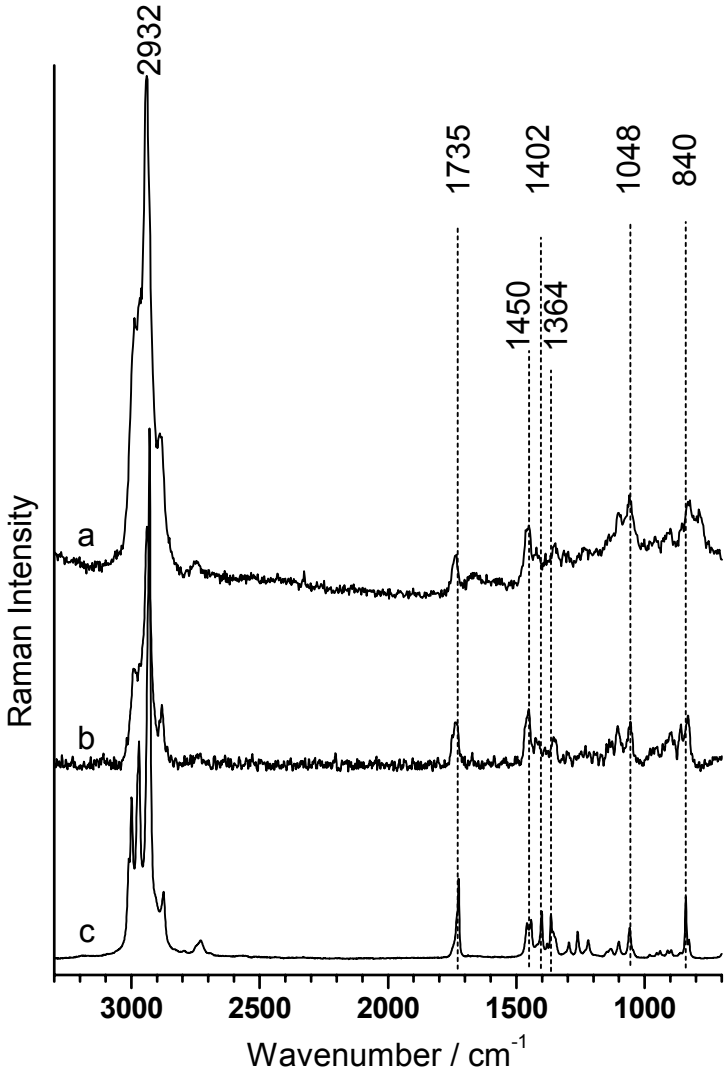

2932
1735
1402
1048
840
1450
1364
a
b
c
Raman Intensity
3000
2500
2000
1500
1000
Wavenumber / cm-1

Figure 4

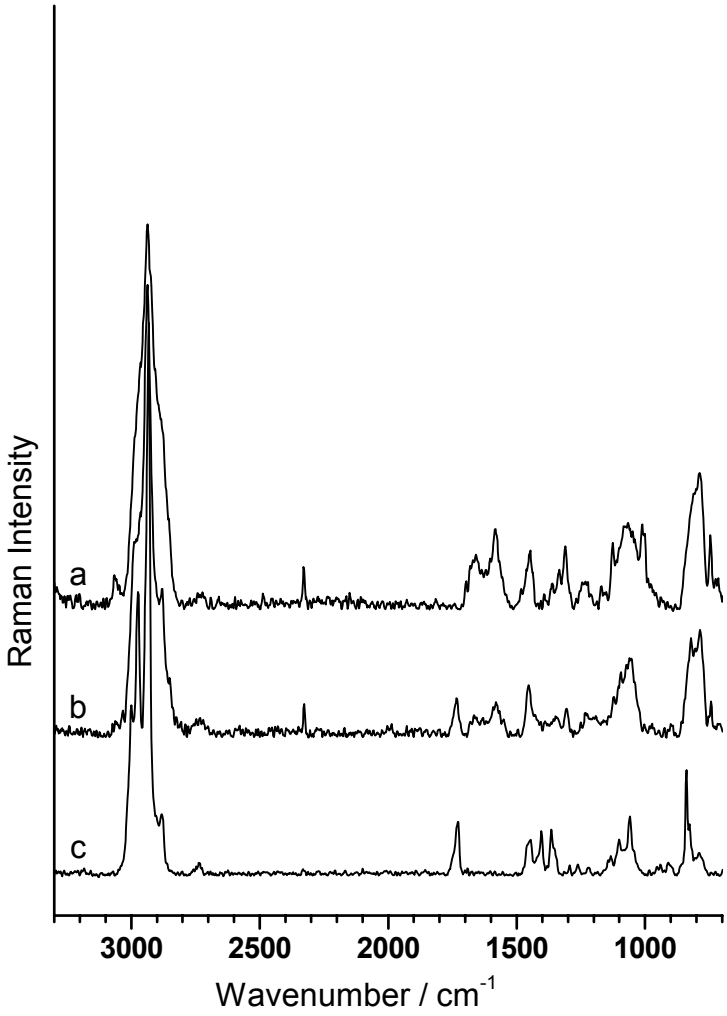
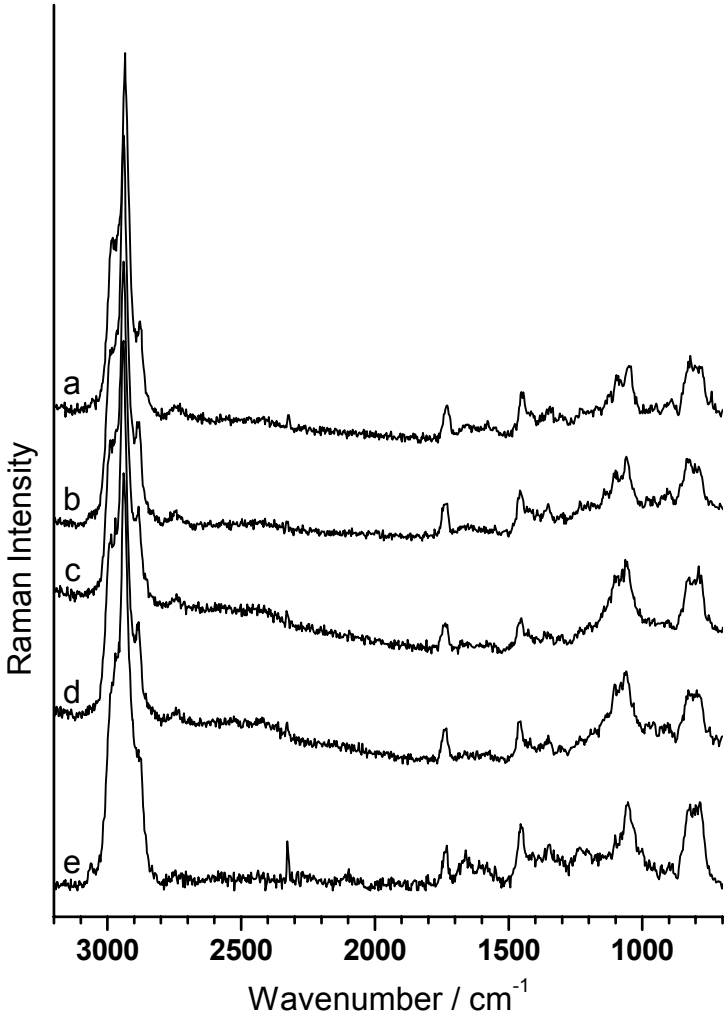

Raman Intensity
a
b
c
3000
2500
2000
1500
1000
Wavenumber / cm-1

Figure 5


Raman Intensity
a
b
c
d
e
3000
2500
2000
1500
1000
Wavenumber / cm-1

General Discussion

Biogeochemical characterization of geochemical barriers

Geochemical barriers were characterized in cooperation with two accompanying diploma theses by Sylvia Löffler (2007) and Sylvia Meißner (2007). At each sampling site two geochemical barriers were identified based on their substantial accumulation of heavy metals and radionuclides.

The glacial sediments at site Gessenwiese (see chapters 1 and 2) were characterized by a sandy texture, pH-values between 4 and 5, high sulfur concentration, low carbon content, and oxidized conditions throughout the sediment profile. In the manganese oxide barrier Cd, Co, and Ni were accumulated, whereas As, U, and also Ni and Co accumulated in the iron oxide barrier. These geochemical barriers were not accompanied by a change in pH, redox state, or grain size distribution, as was reported for metal retaining layers of other sites (Perel'man 1967, McGregor and Blowes 2002, Graupner et al. 2007). Barrier formation might be driven by the capillary rise of contaminated groundwater. Evaporation of the capillary water in the unsaturated zone might lead to oversaturation and precipitation of dissolved elements (Händel 2007). This process would also explain the enrichment of the pore water of the manganese oxide barrier with several metals and sulfur. Metal accumulation appeared to be caused by metal sorption to manganese and iron oxides, respectively, which occurred as a covering on and as a cement between the quartz grains of the sandy sediment matrix. The iron oxide barrier was similar to a patchy, metal-enriched hardpan layer which was described at this site previously, with goethite being the main sorbent in both layers (Carlsson and Büchel 2005). Iron-rich hardpan layers also play a role in contaminant attenuation at other sites (McGregor and Blowes 2002, Graupner et al. 2007). On a molar basis, the manganese oxide barrier appeared to be more effective in metal retention than the iron oxide barrier. A similar predominance of manganese oxides as sorbents for heavy metals was also observed in several other studies (Latrille et al., 2001; Palumbo et al., 2001; Liu et al., 2002; Cornu et al., 2005; Covelo et al., 2007; Dong et al., 2007). However, the dominance of manganese oxides as metal scavengers was surprising, as they are thought to be less important at low pH (Young and Harvey 1992, Lee et al. 2002). In the manganese oxide barrier, also several metals and sulfur peaked in the pore water.

In contrast to the site at the Gessenwiese, the bank soil of the Gessenbach creek had much higher carbon content. In addition, a clear shift in redox conditions was observed within

the soil profile. Two soil horizons, Btlc and Br2, were identified as geochemical barriers (see chapters 1, 3, and 4). In the solid phase of the iron-rich Btlc horizon (formerly named GoII) U, Cu, Zn, Ni, Pb, and As were accumulated mainly due to sorption to iron oxides. Unlike at Gessenwiese, amorphous iron oxides were more important than crystalline iron oxides in Gessenbach soils. The formation of this geochemical barrier was most probably linked to the transition between oxidizing and reducing conditions. The reddish-grey mottled coloration of the Btlc horizon indicated the occurrence of both oxidizing and reducing microniches in close spatial vicinity. This co-occurrence might support iron redox cycling, which could sequester metals within authigenic iron minerals (Cooper et al. 2006). Additionally, metals also peaked in the pore water in this horizon.

In the second geochemical barrier, the sulfate-rich highly reduced Br2 horizon (formerly named GrII), U, Zn, Ni, and Cu were accumulated in the solid phase. This barrier was assumed to be of the H_2S-type, where metals are precipitated as insoluble sulfides (Zelenova et al. 1970). In Br2 soil, uranium was mostly present as U(IV). Sulfate-reducing conditions and high U(IV) sequestration were similarly observed in a black, shallow sediment layer of a freshwater pond located in an open pit uranium mine (Suzuki et al. 2005). Unlike in Btlc, pore water metal concentrations were low in Br2, indicating effective metal retention of the solid phase. Although there was also a manganese-rich horizon, Br1 (formerly named GrI), in the Gessenbach bank soils, manganese oxides in general were less important as metal sorbents here and no particular metal accumulation was observed in this horizon.

Anaerobic microbial redox processes in the geochemical barriers

At the Gessenwiese site, soil respiration was 100 times lower than in Gessenbach bank soils, indicating severe inhibition of microbial activities. Also the total microbial cell counts were low, reaching only about 10^5 - 10^6 cells g sediment $(ww)^{-1}$. Numbers of culturable aerobic microorganisms were in the same range and were only slightly lower in the presence of Ni or Cu, indicating adaptation to high metal concentrations. Altogether, microbial activities seemed to be too low to deplete oxygen and establish anoxic conditions in deeper sediment layers. This was most probably due to the low organic carbon content of the glacial sediment, due to the removal of the top soil material during remediation. Although the area was recontoured with an allochthonous soil (Grawunder et al. 2009), organic carbon content was not higher than 1% g sediment $(dw)^{-1}$ in the deeper layers. After biostimulation the onset of nitrate- and Fe(III)-reduction was observed in anoxic sediment microcosms, indicating the

potential ability of the indigenous microbial community for these processes, but only µmolar amounts of Fe(II) were formed despite the high iron content of the sediment. Altogether, anaerobic microbial redox processes are not likely to occur in situ. Therefore, metal retention and the formation of geochemical barriers were not affected by anaerobic microbial processes in the glacial sediments at Gessenwiese. Carbon limitation rather than heavy metal toxicity seemed to be the major factor affecting microbial activity. However, the impact of aerobic microorganisms, like Fe(II)- or Mn(II)-oxidizing bacteria, was not investigated and can not be ruled out. Such microorganisms oxidize Fe(II) or Mn(II) to Fe(III) or Mn(III)/(IV) that precipitate as Fe(III) or Mn(IV) oxides, which can then act as metal scavengers (Lack et al. 2002, Miyata et al. 2007). Fe(II)-oxidizing microorganisms can use oxygen as electron acceptor at low oxygen concentrations in pH-neutral environments (Emerson 2000). Alternatively, nitrate can serve as an electron acceptor in the absence of oxygen (Straub et al. 1996). Some Fe(II)-oxidizing microorganisms live autotrophically, using CO_2 as a carbon source, and might therefore be able to grow in an environment with low organic carbon like the Gessenwiese sediment (Hallbeck and Pedersen 1990, Sobolev and Roden 2002). Mn(II) can be oxidized in the presence of oxygen and also nitrate could serve as an electron acceptor, although this process has yet to be observed, and only little is known about Mn(II)-oxidizing microorganisms in general (Canfield et al. 2005).

In the Gessenbach bank soils, soil respiration rates were substantially higher than rates in the glacial sediment from Gessenwiese (Löffler 2007). Similar rates were observed previously in the Gessenbach bank soils and rates from uncontaminated bank soils of the Saale river are also in the same order of magnitude (Schmidt et al. 2005). Total cell counts also were much higher than at the Gessenwiese site and geochemical conditions indicated the presence of anaerobic microbial redox processes in both the Btlc and Br2 geochemical barriers. Consequently, the impact of reducing microbial processes on metal dynamics was studied in the bank soil of the Gessenbach.

Within the Btlc horizon, nitrate was depleted and dissolved manganese, supposedly Mn(II), and Fe(II) started to occur in the pore water, indicating the presence of nitrate-, Mn(IV)-, and Fe(III)-reduction. Nitrate was only present in µmolar concentrations. Mn(IV)-reduction also seemed to occur in anoxic Btlc microcosms, although the possibility that manganese was released from sorption sites during reductive dissolution of Fe(III)-oxides can not be ruled out. Total element contents indicated that iron was much more abundant as an electron acceptor than manganese in the Btlc horizon, suggesting that microbial Fe(III)-

reduction was a favourable process. However, without biostimulation, Fe(III)-reduction rates were low or negligible. If Btlc soil was biostimulated by addition of a carbon source, Fe(II)-formation rates were observed, that were at least three times higher than observed in other studies (Akob et al. 2007, Edwards et al. 2007). In the horizons below Btlc Fe(III)-reduction was observed occassionally without biostimulation at similar rates (see chapter 5). Fe(III)-reduction therefore seemed to be inhibited in Btlc soil.

Microaerophilic Fe(II)-oxidizing bacteria have been cultured from the Btlc horizon (Rothhardt 2008). The structure of this horizon, with microniches that might be suitable for different microbial groups, might promote Fe(II)-reoxidation and iron-cycling (Roden et al. 2004, Kappler and Straub 2005). Chemical reoxidation is also possible, due to the partially oxidized conditions in Btlc.

In the Br2 horizon, high sulfur content, low redox potential, and the black color of the soil indicated the dominance of sulfate-reduction. In fact, high *in situ* sulfate-reduction rates were measured both in the Br2 and Br1 horizons, indicating on-going dissimilatory sulfate-reduction in both (see chapter 4). The *in situ* sulfate-reduction rates were slightly higher than what is known from contaminated soils and sediments of the Norilsk mining area (Karnachuk et al. 2005) and were in the range of rates reported from unpolluted freshwater (Holmer and Storkholm 2001, Wind and Conrad 1997) and marine (Sahm et al. 1999, Kristensen et al. 2000) ecosystems. Due to the high reduced sulfur content, Fe(II) might have been formed in Br2 from abiotic reduction coupled to sulfide-oxidation (Thamdrup 2000).

FeRB Communities and their influence on metal dynamics

The impact of Fe(III)-reduction on heavy metal dynamics was especially studied in biostimulated anoxic Btlc-soil microcosms. To investigate the associated active microbial community DNA-SIP was used (see chapter 3). Depending on the carbon source, different microbial populations were detected. A diverse community dominated by *Acidobacteria* (*Geothrix*) and *Firmicutes* (*Pelosinus*) was stimulated by lactate amendment. *Geothrix*, a genus known to reduce nitrate, Mn(IV), Fe(III), and humic acids (Coates et al. 1999), has been detected before in biostimulated uranium-contaminated sediments (Brodie et al. 2006, Luo et al. 2007, Cardenas et al. 2008). Although its specific role in bioremediation is unclear, an indirect reduction of U(VI) by *Geothrix* has been suggested (Cardenas et al. 2008). Members of the *Firmicutes* are able to transfer electrons from fermentation processes to Fe(III) (Lovley 2006). They were suggested to play a role in bioremediation (Akob et al. 2008) due

to their observed reduction and increased sorption of U(VI) (Gao and Francis 2008, N'Guessan et al. 2008). δ-*Proteobacteria* (*Pelobacter*) and γ-*Proteobacteria* (*Dechloromonas* and *Janthinobacterium*) were also detected in the active FeRB community. Members of the genus *Pelobacter* are known to reduce Fe(III) and elemental sulfur, although it was recently suggested that Fe(III)-reduction only occurred indirectly via sulfide production (Lovley et al. 1995, Haveman et al. 2008). The role of *Dechloromonas* and *Janthinobacterium* is not clear, although a contribution to nitrate and probably Fe(III)-reduction is likely (Shivaji et al. 1991, Horn et al. 2005, Chung et al. 2006, Hashidoko et al. 2008).The genus *Dechloromonas* has been observed before in uranium-contaminated soil microcosms (Akob et al. 2008, Mohanty et al. 2008). No clear shift to a sulfate-reducing community could be observed at a later time point of the SIP experiment, probably due to the ongoing reduction of high amounts of Fe(III) during sulfate-reduction.

The addition of ethanol stimulated the activity of *Geobacter* in particular, a genus of the δ-*Proteobacteria* which is important in Fe(III)- and U(VI)- reducing communities. This is in good agreement with other studies (Holmes et al. 2002, Anderson et al. 2003, North et al. 2004, Akob et al. 2008, Mohanty et al. 2008). *Acidobacteria* (*Geothrix*) and *Firmicutes* (*Desulfosporosinus*) were detected in lower abundances early during incubation, but later on became important members of the community. *Desulfosporosinus* species are able to reduce sulfate, Fe(III), and As(V) (Spring and Rosenzweig 2006, Ramamoorthy et al. 2006). These organisms may have been involved in As solubilization and Fe(III)-reduction at the beginning of the experiment and may also have contributed to sulfate-reduction. *Geothrix* can utilize acetate but not ethanol as electron donor, which explains its low abundance at an early time point during ethanol consumption (Coates et al. 1999). However, *Geothrix* might have contributed to Fe(III)-reduction later during the incubation period.

In general, biostimulated Fe(III)-reduction facilitated the release of metals in anoxic soil microcosms, while subsequent sulfate-reduction was mostly associated with metal immobilization. Most metals were supposedly released from sorption sites during reductive dissolution of Fe(III) oxides (Francis and Dodge 1990, Cambier and Charlatchka 1999). Our data indicated that some of the metals, especially Cd and Cu, might also be released from manganese oxides. Most FeRB are also able to reduce Mn(IV) (Canfield et al. 2005); therefore, it is likely that FeRB communities also affect metals bound to manganese oxides. As(V) can also be reduced by FeRB and, consequently, As release might be due to direct reduction as well as reductive dissolution of sorbents (Islam et al. 2005, Tufano et al. 2008).

Despite the low Fe(III)-reducing activity without biostimulation, the heavy metal peaks observed in the pore water of Btlc might indicate heavy metal release due to reductive dissolution of iron oxides. Alternatively, these metals might also be transported to this site by seepage water. Metal immobilization during sulfate-reduction was probably due to formation of metal sulfides with Co, Ni, Zn, Cd, and As (Ehrlich 1999, Krumholz et al. 2003, O'Day et al. 2004, Mandal et al. 2006, Gramp et al. 2007), although precipitation onto newly formed iron phases can not be excluded (Cooper et al. 2006). Since As concentrations were below those previously reported to facilitate arsenic sulfide precipitation (O'Day et al. 2004), formation of As-bearing pyrite might also have occurred (Saunders et al. 2008).

Surprisingly, uranium was also released during the period of microbial Fe(III)-reduction. FeRB, especially *Geobacter*, are known to remove U(VI) from solution through precipitation of the insoluble U(IV) mineral phase uraninite [UO_2(s)] (Gorby and Lovley 1992, Lovley 1995, Wall and Krumholz 2006). Immobilization of uranium during Fe(III)-reduction is observed for other uranium-contaminated sites in the US such as Rifle (CO) and Oak Ridge (TN), (Anderson et al. 2003, Akob et al. 2008). In the solid phase of the Btlc-soil about 30% of the U was associated with amorphous Fe(III)-oxides and 81% of the U was U(VI) as detected by XANES measurement. U(VI) can form soluble complexes with Ca and carbonate that would be persistent to enzymatic reduction (Brooks et al. 2003, Neiss et al. 2007). The formation of such complexes, might be insignificant due to the lack of carbonates in the soil and the lack of CO_2 in the microcosm headspace at the beginning of the incubation. However, U(VI), mainly present as uranyl complexes, strongly adsorb to iron oxides (His and Langmuir 1985). U(VI) likely was sequestered in the Btlc horizon during Fe remineralization processes occurring at the redox transition boundary. Our results suggest that solid phase associated U was not enzymatically reduced by FeRB, but was released during reductive dissolution of Fe(III)-oxides. The partial decrease of U started with the onset of sulfate-reduction, although a contribution of FeRB cannot be ruled out. Several sulfate-reducing bacteria reduce U(VI) enzymatically (Wall and Krumholz 2006), although only one was reported to gain energy for growth from this process (Tebo and Obraztsova 1998). Additionally, sorption of U to metal sulfides, such as pyrite, might be possible (Wersin et al. 1994). Comparative spectra demonstrated that no UO_2(s) was present but apparently a sorbed complex of U(IV). During reoxidation of the microcosms reoxidized U again is scavenged in newly formed Fe(III) and Mn oxides (Lack et al. 2002, Webb et al. 2006), confirming the importance of iron oxides for U retention in the Btlc horizon.

The metal retention observed during sulfate-reduction was confirmed by studies with Br2 soil. Sulfate-reducing enrichment cultures originating from horizon Br2 precipitated Ni and Co, supposedly by metal sulfide formation (see chapter 4). Those metals also precipitated in anoxic Br2-soil microcosms independently of biostimulation, suggesting on-going, *in situ* retention of metals by sulfate-reducing activity (Fortin et al. 1994, Krumholz et al. 2003). However, concentrations of dissolved Cu, Zn, and As did not decrease consistently in all experiments. This was in contrast to previous work that shows that Zn, Cu and As typically form sulfides under sulfate-reducing conditions (Newman et al. 1997, Labrenz et al. 2000, Southam 2000). As(VI)-reducing bacteria, such as *Desulfosporosinus auripigmenti*, can contribute to the observed increase in soluble arsenic (Newman et al. 1997). In contrast to the decreasing soluble U concentrations observed during sulfate-reduction in Btlc-microcosms, soluble U concentrations increased under sulfate-reducing conditions in Br2-microcosms. This also contrasted with previous studies (Abdelouas et al. 2000, Wu et al. 2007), although a few report an increase in uranium concentration during sulfate-reduction in uranium-contaminated aquifers (Anderson et al. 2003) or in pure cultures of *Desulfovibrio desulfuricans* G20 (Sani et al. 2005).

Metal tolerance of FeRB

While total cell counts in the bank soil of the Gessenbach were in a normal range with up to 10^9 cells g (fresh weight soil)$^{-1}$ (Horner-Devine et al. 2004, Bauer 2007), numbers of culturable Fe(III)-reducing microorganisms were relatively low and were further reduced by three orders of magnitude in the presence of heavy metals. The metal concentrations used corresponded to the mobile and replenishable fractions, as obtained by sequential extraction (Löffler 2007). Those fractions are considered to be bioavailable, and *in situ* metal concentrations could therefore inhibit Fe(III)-reduction. Pore water heavy metal concentrations were much lower and an intense solubilization process would be necessary to liberate such high metal concentrations. However, microbial abundance might have been underestimated by MPN-analysis, due to cultivation conditions. With qPCR 10^6 *Geobacteraceae* genome copies g soil (ww)$^{-1}$ were detected, which was 0.02 – 0.04% of the total community in Btlc soil. Those copy numbers were low compared with those obtained by qPCR by other authors (Holmes et al. 2002, Wan et al. 2005) for sediments without *in situ* Fe(III)-reduction, probably due to metal toxicity. A biological effect of the heavy metals present in Gessenbach bank soils was also indicated in previous studies, where an adaptation

of spore forming *Actinobacteria* to metal contamination was observed and heavy metal tolerant strains were isolated (Schmidt et al. 2005). Growth of *Streptomyces mirabilis* isolates can still be observed on plates containing more than 100 mM Ni or Zn (Schmidt et al. 2009). Those organisms protect themselves and neighbouring plants from inhibitory effects of heavy metals by the production of siderophores (Dimkpa et al. 2008, Dimkpa et al. 2009). However, the bioavailable metal concentrations present in the plates were not measured and, although a minimal medium was used for these experiments, bioavailable metal concentrations might be lower, due to precipitation with media components.

In Fe(III)-reducing enrichment cultures, the activity of FeRB is already inhibited at µmolar concentrations of Ni, Cu, Co, and Zn, comparable to low metal tolerances reported for *Shewanella* and *Clostridium* species (Francis and Dodge 1988, Stone et al. 2006, Toes et al. 2008, see chapter 5). In contrast to these low metal tolerances, no inhibition of hematite reduction by *Shewanella putrefaciens* was observed up to 1.63 mM Ni in a study by Paul and Stone (2009). However, the bioavailability of metals in cultures is strongly decreased due to interaction with media components. In most previous studies dissolved metal concentrations were not determined and therefore, metal tolerances might be lower than previously reported. The effect of the medium on metal toxicity was demonstrated in a study by Toes et al. (2008), where Cu tolerance is between 75 and 750 µM depending on nutrient load and the presence of manganese oxides also reduces the toxicity of Cu. However, considering dissolved metal concentrations, similarly low metal tolerance has been observed in Fe(III)-reducing communities of different soils. In a variety of soils solution concentrations of 2.8 – 1503 µM Cd inhibit 50% of Fe(III)-reduction (Welp and Brümmer, 1997). The toxic concentration of Cd in the soil depends on the soil type and associated parameters like organic matter content, sorption abilities, and pH.

The study by Welp and Brümmer (1997) demonstrates the significance of low metal concentrations for Fe(III)-reducing activities in different soils. In this thesis, the low or negligible Fe(III)-reduction rates observed in non-biostimulated Btlc microcosms also indicated a toxic effect of metal contaminants. However, Btlc-pore water metal concentrations were tolerated by the Fe(III)-reducing enrichment cultures, but not metal concentrations present in the bioavailable fraction of the soil solid phase. Toxicity of solid phase metals was mainly due to Cu, Ni, and partially Zn. Altogether, microbial Fe(III)-reduction in soils might be already affected at low metal concentrations and depend on a metal scavenging process or a sufficient carbon source for detoxification. Iron oxides are among the most abundant

minerals in soils and undergo various interactions with other soil components (Canfield et al. 2005, Kappler and Straub 2005). Consequently, FeRB influence processes like organic carbon mineralization, bioavailability of nutrients and toxic elements, and contaminant behaviour and degradation in the soil (Lovley 2006). Knowledge about the impact of low concentrations of heavy metal contaminants on the activity of FeRB is important to evaluate the potential for natural attenuation and to assess risks for soil function.

Geobacter and *Firmicutes* were detected in a clone library of metal tolerant enrichment cultures (see chapter 5). The genus *Geobacter* is generally thought to play an important role in microbial Fe(III)-reduction at neutral pH, as it has been identified in cultivation-based as well as cultivation independent approaches in a wide range of environments (Lovley et al. 2004). In ethanol-biostimulated bank soil of the creek Gessenbach *Geobacter* was also a dominant active FeRB. *Firmicutes* were important as well during Fe(III)-reduction in treatments amended both with ethanol and lactate. However, this clone library included organisms from a non-inhibited culture with very low metal content (1.1 µM Cu added). Therefore, some of the organisms identified might not be metal-tolerant. Sequencing the main bands of DGGE community fingerprints revealed a clear dominance of *Firmicutes* in a Zn-tolerant culture. Those *Firmicutes* were related to fermenters of the genus *Sedimentibacter* and the order *Clostridiales*. *Sedimentibacter* strains are found frequently in dechlorinating enrichments (Breitenstein et al. 2002, van Doesburg et al. 2005, Cheng et al. 2010). The *Clostridiales* related clone shared a high 16S rRNA gene sequence identity with a clone obtained from a tar oil-impacted aquifer (Winderl et al. 2008). Additionally the isolate, DA-1 originating from Cd or Cu amended enrichments, was closely related to *Desulfosporosinus lacus*. This genus has also been detected in biostimulated Btlc-microcosms, where it may have been involved in sulfate- and Fe(III)-reduction (Spring and Rosenzweig 2006, Ramamoorthy et al. 2006). Members of this genus have been detected in various contaminated sites, including radionuclide contaminated sediment (Robertson et al. 2001, Suzuki et al. 2002, Shelobolina et al. 2003, Nevin et al. 2003). *D. lacus* tolerates 2 mM As, but is inhibited by 10 mM As, 10 µM Cd, 0.4 mM Cr, and 10 µM Zn (Ramamoorthy et al. 2006). These concentrations are similar to dissolved metal concentrations in the enrichments from which DA-1 was isolated from (23 µM Cd or 14 µM Cu). Altogether, these data suggested, that DA-1 probably reduces Fe(III) in the enrichment cultures.

However, most *Firmicutes* are only able to transfer part of the electrons from fermentation to Fe(III) (Francis and Dodge 1988). In a former study the importance of a

fermentative *Clostridium* for microbial reduction of Cu-contaminated ferric oxide was demonstrated (Markwiese and Colberg, 2000). Although the fermenter reduces only a minor amount of the iron, it seems to ameliorate metal toxicity towards Fe(III)-reducers by binding dissolved Cu. Fe(III)-reduction only starts after dissolved Cu concentrations decrease to 5 ppb. Therefore, although their Fe(III)-reducing potential is low compared to other FeRB, *Firmicutes* might be important for Fe(III)-reduction in metal contaminated environments. The importance of *Firmicutes* for *in situ* Fe(III)-reduction might then be underestimated, especially when the low metal tolerance of FeRB is considered.

The heavy metal tolerance of an acidophilic Fe(III)-reducing model organism, *Acidiphilium cryptum* JF-5, was also determined. This work was done by Stefan Gischkat during a laboratory course under my supervision. High metal tolerances of acidophilic microorganisms, including *Acidiphilium* have been reported before (Dopson et al. 2003). *Acidiphilium* species were isolated from acidic environments, such as acid mine drainage sites (Küsel et al. 1999, Hallberg and Johnson 2001). In these environments most metals are highly soluble and high bioavailable concentrations may have favored the selection of metal resistant

Table 1: Intensity of Fe(II)-formation from dissolved $Fe_2(SO_4)_3$ and duration of lag phases in Fe(III)-reducing cultures of *Acidiphilium cryptum* JF-5 grown in the presence of metals (data were obtained by Stefan Gischkat during a laboratory course under my supervision).

Treatment	Concentration added [mM]	Duration of lag phase [d]	Fe(II)-formation-rates[a]
Control	No metal added	2	+++
$KCrO_4$	0.1	2	+++
	0.5	16	++
	1.0	no Fe(III)-reduction within 59 days	
$CuCl_2$	10	2	+++
	20	5	++
	100	no Fe(III)-reduction within 59 days	
$NiCl_2$	10	2	+++
	100	no Fe(III)-reduction within 31 days	
$ZnCl_2$	10	2	++
	100	13	+
	500	no Fe(III)-reduction within 31 days	

[a] +++/++/+ Fe(II)-formation rates: 4-6/ 2-4/ 0-2 μmol ml^{-1} d^{-1}.

microorganisms. It has been demonstrated that the high resistance of *A. symbioticum* KM2 to Cd and Zn is plasmid mediated (Mahapatra et al. 2002). Plasmids are common vectors for resistance genes and enable otherwise sensitive organisms to thrive in the presence of toxic elements or compounds, like antibiotics (Silver 1992). The heavy metal concentrations tolerated by *A. cryptum* JF-5 (see table 1) were in a similar range as reported before by Dopson et al. (2003). Fe(II)-formation by strain JF-5 was not inhibited by 0.1 mM Cr, 10 mM Cu, 10 mM Ni, or 10 mM Zn. Fe(II)-formation rates were reduced and the lag phase was extended in the presence of 0.5 mM Cr, 20 mM Cu, or 100 mM Zn, and no Fe(II)-formation could be observed any more at 1 mM Cr, 100 mM Cu, 100 mM Ni, or 500 mM Zn. Fe(III) was also reduced in the presence of 700 mM Cd, added as $CdCl_2$, but not with 1000 mM Cd (data not shown). Due to the low culture pH, all metals added were dissolved in the culture medium, as confirmed by ICP-OES measurements. Therefore, *A. cryptum* JF-5 might also serve as a model organism for metal tolerance in acidophilic microorganisms.

Raman spectroscopic measurements of *Acidiphilium cryptum* JF-5

Raman spectroscopic identification of *A. cryptum* JF-5 was done in cooperation with Valerian Ciobotă (Institute of Physical Chemistry). First, the impact of storage compounds on microbial identification had to be evaluated (see chapter 6). The formation of storage compounds like polyhydroxybutyrate (PHB) is common in microbial metabolism (Anderson and Dawes 1990) and is also known for *Acidiphilium* (Xu et al. 2010). Our experiments demonstrated that JF-5 formed amorphous PHB during exponential growth. In contrast, PHB was present as crystalline form during the stationary phase. With increasing PHB content of the cells, Raman signals from other cell components, like nucleic acids and proteins, decreased in intensity, which likely affects identification. Best results were obtained if cells from the late exponential growth phase were used for the measurements. In this phase, accumulation of amorphous PHB is still on-going and the Raman signals of proteins and nucleic acids were not yet completely obscured. In the stationary phase identification was not possible. Spectra of different PHB producing bacteria could be identified with a recognition rate of 90.1%. Therefore, Raman spectroscopy could be a tool for microbial identification, even in the presence of storage compounds like PHB.

Secondly, Raman spectroscopy was applied to differentiate between cells of the same strain grown under different conditions (table 2). It was possible to differentiate between cells grown with different electron acceptors, like oxygen, soluble $Fe_2(SO_4)_3$, and solid phase

Fe(III)-sources. Differentiation between two solid phase Fe(III)-sources, $Fe(OH)_3$ and hematite, was not possible. These results suggested the existence of different electron transfer pathways to dissolved and solid phases of Fe(III). The reduction of solid phase Fe(III) requires either direct contact between cell and mineral or the use and probably production of chelators or electron shuttles (Reguera et al. 2005, Lovley 2006). The different spectra may reflect the presence of additional proteins involved in catalyzing access to Fe(III).

Table 2: Recognition rates for the identification of *Acidiphilium cryptum* JF-5 grown under different culture conditions. Values are given for each group of treatments. Treatments within one group are separated by "/". Good identification rates are printed in bold letters. The values were obtained in cooperation with Valerian Ciobotă (Institute of Physical Chemistry).

Groups of treatments	Recognition rate [%]
$Fe_2(SO_4)_3$ / O_2	**94.5**
$Fe_2(SO_4)_3$ / $Fe_2(SO_4)_3 + O_2$	77.4
$Fe(OH)_3$ / $Fe(OH)_3 + O_2$ / O_2	**94.9**
soluble Fe source / insoluble Fe sources	**92.2**
hematite[a] / $Fe(OH)_3$	85.3
$Fe_2(SO_4)_3 + O_2$ / $Fe(OH)_3 + O_2$	**98.1**
with heavy metals / without heavy metals	84.0
Cd / Ni / Cr / Cu[b]	**91.3**
Ni / Zn[c]	**100.0**
0.1 µM Ni / 10 µM Ni / 1 mM Ni	**93.1**
0.1 µM Cu / 10 µM Cu / 1 mM Cu / 5 mM Cu	**98.1**
0.1 µM Cd / 10 µM Cd / 1 mM Cd	75.5
10 mM Zn / 20 mM Zn	**92.5**

[a] Goethite was added to the cultures as electron donor, but was transformed to hematite during autoclaving. The hematite was identified by Raman spectroscopy.
[b] This analysis included spectra of cultures amended with the following metal concentrations: 0.1 µM, 10 µM, 1 mM of $NiCl_2$ and $CdCl_2$; 0.1 µM, 10 µM, 1 mM, 5 mM of $CuCl_2$; 0.1 µM, 10 µM of $KCrO_4$.
[c] This analysis included spectra of cultures amended with the following metal concentrations: 10 mM $NiCl_2$; 10 mM, 20 mM $ZnCl_2$.

In addition to the presence of electron acceptors, the presence of toxic compounds, like heavy metals, might also induce changes in the cell physiology through stress response and resistance mechanisms. With Raman spectroscopy it was not possible to differentiate between cells grown in the presence or absence of metals (table 2). However, the metal

concentrations used were low compared to the metal tolerances observed for *A. cryptum* JF-5 (see above), and may not have been sufficient to induce a stress response. Therefore, higher metal concentrations which clearly have an effect on the activity of JF-5 need to be tested. However, it was possible to differentiate between cells grown in the presence of different heavy metals and also at different metal concentrations. Altogether, Raman spectroscopy may prove to be a useful tool for differentiating between cells with different growth histories, independent of the presence or absence of storage materials. Until now, the potential of Raman spectroscopy for such applications has been reported only for cells which contain no storage granules (Harz et al. 2005, Schmid et al. 2009).

Implication for the Gessenbach and its catchment area

At the Gessenwiese, metal content of the sediment was elevated compared to background values, but with the exception of As, element concentrations were below regulated values from the German regulation for soil in recreational areas (BBodSchV,1999), which is consistent with the current use of this area. In the pore water Ni, As, and Cd were present at high concentrations, indicating the possible transport of these metals in the groundwater. However, the results of this thesis indicate that both geochemical barriers likely prevent contaminant mobility in the aquifer. However, microbial activity was limited by carbon availability, and reductive processes could be stimulated by the addition of a carbon source, demonstrating the potential ability of the indigenous microbial community for anaerobic processes, although they are not likely to occur *in situ*. However, after development of a more dense vegetation and an increase in organic carbon content of the sediment in the next decades, nitrate-and Fe(III)-reducing activities might be initiated and metals could be released, similarly as in soil from the Btlc horizon at the Gessenbach. Therefore, Gessenwiese might remain a source of contaminants in the future. Contaminants likely could be transported to the Gessenbach and ultimately influence creek water quality.

In the bank soil of the Gessenbach a release of metals might be facilitated by the activity of FeRB in the Btlc horizon. However, Fe(III)-reduction appeared to be inhibited *in situ*, and metal solubilization might therefore be low. In the pore water of the bank soil Ni concentrations highly exceed drinking water standards and also Cd concentrations are elevated, indicating the risk of metal discharge. Metals released from the Btlc horizon likely do not enter directly into the creek water as there is no direct contact between this horizon and the creek water table. Instead, metals would likely be transported into deeper soil horizons

and scavenged in the Br2 horizon. Sequestration of Ni in the Br2 horizon indicates natural attenuation that may decrease the downstream transport of Ni.

Consequently, monitoring of creek water quality, as currently conducted by the Wismut GmbH, and perhaps treatment of the creek water will be important to prevent hazards for grassland usage and villages in downstream areas. The contaminant of most concern is Ni, as its concentration greatly exceeds drinking water standards, also in the creek water (Wismut 2006). U concentrations in the creek water also still exceed values proposed for drinking water standards (Konietzka et al. 2005), but the excess is not as high as for Ni and pore water concentrations are generally acceptable.

Conclusions

With respect to hypothesis I "FeRB support the formation of metal-retaining iron- and manganese-rich geochemical barriers in metal-contaminated soil and sediment." it can be stated that FeRB activity generally was associated with metal release. Therefore, hypothesis I should be rejected. FeRB likely do not facilitate natural attenuation in the bank soils of the Gessenbach, but instead might cause a release of metals including uranium, which is in contrast to former studies (Anderson et al. 2003, Akob et al. 2008). Biostimulation of FeRB, as it was suggested for uranium remediation in other sites in the US, would not be appropriate at this site. However, it remains unknown how cyclic reduction and oxidation of iron, which can cause metal retention (Cooper et al. 2006) and probably occurs in the Btlc horizon, would affect metal retention. In this thesis during single reoxidation of highly reduced Btlc microcosms Ni, Cu, and Cd were released, while U seemed to be sequestered again. However, reoxidation from less reduced conditions *in situ* might have a different effect on metal retention. Therefore, more research concerning Fe(II)- and Mn(II)-oxidation and iron cycling is needed.

Hypothesis II, "FeRB indigenous to a metal-contaminated soil are highly tolerant to heavy metal stress." also should be rejected for the neutrophilic FeRB investigated in this thesis. This work demonstrates that microbial Fe(III)-reduction may be sensitive to low metal concentrations and might depend on a metal scavenging mechanism or a sufficient carbon source for detoxification. This would be of major environmental concern, as Fe(III)-reducing microorganisms play an important role in geochemical cycles of iron and other metals and also influence other major processes in soils (Lovley 2006). In contrast, acidophilic FeRB, like *Acidiphilium cryptum* JF-5, could tolerate higher metal concentrations.

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Summary

Iron oxides are important sorbents for metals in soils and have a major impact on soil processes due to various interactions with other soil components. Fe(III)-reducing bacteria (FeRB) can facilitate the release of sorbed metals by reductive dissolution of iron oxides, change the speciation and solubility of metals by direct or indirect reduction, and therefore influence metal mobility in soils. Thus, the activity of FeRB might affect the formation and stability of geochemical barriers, local epigenetic zones where element migration conditions are distinctly altered and therefore substantial accumulation of metals can occur. Understanding the dynamics of metals and the associated microbial communities is necessary for evaluating risks for pristine sites. The ability for Fe(III)-reduction is widespread within the *Eubacteria* and the *Archaea*, but the identification of these organisms remains difficult due to the absence of a common functional gene.

This thesis is part of the Graduate Research School 1257 "Alteration and element mobility at the microbe mineral interface". The main aim of this thesis was to investigate the impact of FeRB on heavy metal retention and the effect of heavy metals on the activity of FeRB. Two sampling sites with geochemical barriers were selected, both located in the former uranium mining district Ronneburg (Thuringia, Germany). One site, Gessenwiese, is located on the basement area of a former leaching heap, where elevated heavy metal concentrations remain in the glacial sediments, due to infiltration of the leachate during leaching operations. The second site, Gessenbach, which is the main drainage system for the Gessenwiese area, received metal-containing seepage water and surface runoff and metals enriched in the bank soils.

At the Gessenwiese two metal accumulating sediment layers were identified by total and sequential extraction of the sediment solid phase. Metals accumulated in manganese and crystalline iron oxides. The formation of the geochemical barriers was probably due to capillary rise of contaminated groundwater. Microbial abundance and activity were generally low at this site and anaerobic processes are unlikely to occur, due to oxidized conditions in the sediment. Altogether, microbial activity was limited by low carbon availability and did likely not affect metal retention.

In the Gessenbach bank soils metals accumulated in two soil horizons, Btlc and Br2. The iron-rich Btlc horizon was characterized by a transition between oxidized and reduced conditions and geochemical data indicated an importance of Fe(III)-reduction. However,

Fe(III)-reduction rates were low in non-biostimulated anoxic soil-slurries, indicating inhibition of Fe(III)-reduction *in situ*. Metals were mainly bound to amorphous iron oxides, as indicated by sequential extraction, and metal accumulation is probably linked to the transition between redox conditions. In the sulfate-rich, reduced Br2 horizon high sulfate-reduction rates indicated on-going sulfate-reduction and metal retention might be caused by metal sulfide formation.

In biostimulated Btlc-soil slurries, soluble metal concentrations increased during Fe(III)-reduction, likely due to a release from sorption sites during reductive dissolution of Fe(III)-oxides. Unexpectedly U was released, which is in contrast to other studies. During subsequent sulfate-reduction soluble metal concentrations, including U, decreased. Reduction of U(VI) likely occurred during sulfate-reduction. The active Fe(III)-reducing population, which was identified by using a stable isotope probing approach, was dominated by δ-*Proteobacteria* (*Geobacter*) in ethanol amended microcosms. A more diverse community, dominated by *Acidobacteria* (*Geothrix*) and *Firmicutes* (*Pelosinus*) was stimulated by the addition of lactate. In Br2-soil slurries Ni and Co precipitated during sulfate-reduction, in agreement with the observation in Btlc soil, but U concentrations increased.

FeRB indigenous to Btlc soil generally tolerated only µmolar metal concentrations in enrichment cultures. Fe(III)-reduction might be inhibited by soil concentrations of Cu and Ni, although pore water concentrations were tolerated. A clone library of metal amended cultures was dominated by δ-*Proteobacteria* (*Geobacter*) and *Firmicutes*. Sequencing of DGGE bands demonstrated a dominance of *Firmicutes* in Zn-tolerant cultures and an isolate related to *Firmicutes* was obtained from Cu and Cd amended cultures. By qPCR 10^6 *Geobacteraceae* genomes per g fresh weight soil were detected, which is 0.02 to 0.04% of the total microbial community in Btlc soil. Those results indicate that *Firmicutes* might be important for Fe(III)-reduction in metal contaminated enviroments. In contrast to this, an acidophilic model FeRB, *Acidiphilium cryptum* JF-5, tolerated high metal concentrations in a mmolar range.

The FeRB *A. cryptum* JF-5 could be identified by means of Raman spectroscopy, although the intensity of the Raman signals was decreased due to the formation of polyhydroxybutyrate (PHB) as a storage compound. With Raman spectroscopy it was possible to differentiate between aerobic and anaerobic metabolism and between dissolved and solid phase Fe(III) as electron acceptors, suggesting the existence of different electron transfer pathways to both Fe(III) phases. Differentiation of cells grown in the presence of different heavy metals and at different metal concentrations was also possible.

Altogether, Fe(III)-reducing communities in horizon Btlc might facilitate the release of metals including U to groundwater. Those metals might be transported into deeper soil horizons, and might be scavenged in horizon Br2. However, the possible effect of iron redox cycling on metal retention was not investigated in this thesis. In the future, after carbon availability increased, reducing processes might also be initiated at Gessenwiese, and this site might remain a source of contaminants due to metal release during Fe(III)-reduction. This thesis also provides evidence that microbial Fe(III)-reduction is sensitive even to low metal concentrations and might depend on a metal scavenging mechanism or a sufficient carbon source for detoxification. Therefore, soil function might be disturbed already by low amounts of metal contaminants.

Zusammenfassung

Eisenoxide sind wichtige Metall-Sorbenten in Böden und haben einen wesentlichen Einfluss auf Bodenprozesse durch Wechselwirkungen mit einer Vielzahl anderer Bodenbestandteile. Fe(III)-reduzierende Bakterien (FeRB) können durch die reduktive Auflösung von Eisenoxiden die Freisetzung von sorbierten Metallen verursachen, die Speziation von Metallen durch direkte und indirekte Reduktion verändern und dadurch die Verfügbarkeit von Metallen in Böden beeinflussen. Die Bildung und Stabilität von geochemischen Barrieren, lokalen epigenetischen Zonen, in denen durch veränderte Bedingungen für die Migration von Elementen beträchtliche Anreicherungen von Metallen auftreten können, könnte daher durch die Aktivität von FeRB beeinflusst werden. Das Verständnis der Metall-Dynamik und der damit verbundenen mikrobiellen Gemeinschaften ist notwendig, um Risiken für nicht kontaminierte Flächen abschätzen zu können. Die Fähigkeit zur Fe(III)-Reduktion ist innerhalb der *Eubacteria* und *Archaea* weit verbreitet, aber durch das Fehlen eines gemeinsamen funktionellen Gens ist die Identifizierung dieser Organismen schwierig.

Diese Arbeit ist Teil des Graduiertenkollegs “Alteration und Element-Mobilisierung an Mikroben-Mineral-Grenzflächen”. Das Hauptziel dieser Arbeit war es, den Einfluss von FeRB auf die Retention von Schwermetallen und die Auswirkungen von Schwermetallen auf die Aktivität von FeRB zu untersuchen. Hierfür wurden zwei Probenahmestellen im ehemaligen Uran-Bergbaugebiet Ronneburg (Thüringen, Deutschland), bei denen geochemische Barrieren auftreten, ausgewählt. Die Probenahmestelle Gessenwiese befindet sich auf der Aufstandsfläche der ehemaligen Laugungshalde Gessenhalde. Die glazialen Sedimente dieser Fläche weisen erhöhte Schwermetall-Konzentrationen auf, welche durch das Eindringen von Sickerwasser während des Betriebs der Halde verusacht wurden. Der Gessenbach, die zweite Probenahmestelle, entwässert die Gessenwiese. Daher ist er durch schwermetallhaltige Sicker- und Oberflächenwässer beeinflusst und im Uferboden haben sich Schwermetalle angereichert.

Auf der Gessenwiese wurden durch Total- und sequenzielle Extraktion der Sediment-Festphase zwei geochemische Barrieren identifiziert, in denen Metalle in Mangan- und Eisen-oxiden angereichert waren. Die Bildung dieser Barrieren könnte durch kapillar aufsteigendes Grundwasser verursacht worden sein. Die Abundanz und Aktivität von Mikroorganismen im Sediment der Gessenwiese war generell niedrig und das Auftreten anaerober Prozesse ist

durch die oxidierten Bedingungen unwahrscheinlich. Insgesamt war die mikrobielle Aktivität Kohlenstoff-limitiert und beeinflusste wahrscheinlich nicht die Retention der Metalle.

Im Uferboden des Gessenbachs reicherten sich Metalle in zwei Horizonten, Btlc und Br2, an. Der eisenreiche Btlc-Horizont war durch den Übergang von oxidierten zu reduzierten Bedingungen geprägt. Ein großer Teil der Metalle war an amorphe Eisenoxide gebunden. Die Metall-Akkumulation steht möglicherweise in Zusammenhang mit dem Wechsel der Redox-Bedingungen. Die geochemischen Daten wiesen auf Fe(III)-Reduktion als wichtigen Prozess hin. Die Fe(III)-Reduktionsraten hingegen waren in nicht-biostimulierten anoxischen Boden-Mikrokosmen niedrig, was auf eine Hemmung der Fe(III)-Reduktion *in situ* hinweist. Im sulfatreichen reduzierten Br2-Horizont deuteten hohe Sulfat-Reduktionsraten auf das Auftreten von Sulfat-Reduktion *in situ* hin und die Rückhaltung von Metallen ist wahrscheinlich durch die Bildung von Metallsulfiden verursacht.

In biostimulierten Btlc-Mikrokosmen stieg die Konzentration gelöster Metalle während der Fe(III)-Reduktion an, wahrscheinlich durch Desorption während der reduktiven Auflösung von Fe(III)-Oxiden. Im Gegensatz zu anderen Untersuchungen wurde auch Uran freigesetzt. Während der nachfolgenden Sulfat-Reduktion nahm die Konzentration gelöster Metalle, darunter auch Uran, ab. Eine Reduktion von U(VI) fand wahrscheinlich erst während der Sulfat-Reduktion statt. Die aktiven Fe(III)-reduzierenden Gemeinschaften wurden mit Hilfe von *stable isotope probing* identifiziert. In Ethanol-biostimulierten Mikrokosmen wurde die Population von δ-*Proteobacteria* (*Geobacter*) dominiert. Eine vielfältigere Gemeinschaft, dominiert von *Acidobacteria* (*Geothrix*) und *Firmicutes* (*Pelosinus*), wurde nach der Biostimulation durch Laktat identifiziert. In Br2-Mikrokosmen wurden Ni und Co ebenfalls während der Sulfat-Reduktion präzipitiert, aber Uran wurde auch hier freigesetzt.

Im Btlc Horizont vorkommende FeRB tolerierten in Anreicherungskulturen nur µmolare Metall-Konzentrationen. Während die Porenwasser-Konzentrationen toleriert wurden, könnte die Fe(III)-Reduktion durch in der Boden-Festphase enthaltenes Cu und Ni gehemmt werden. Im Gegensatz dazu tolerierte das acidophile FeRB *Acidiphilium cryptum* JF-5 mmolare Metallkonzentrationen. Die mikrobielle Gemeinschaft der in Gegenwart von Metallen gewachsenen Kulturen wurde in einer Klonbibliothek von δ-*Proteobacteria* (*Geobacter*) und *Firmicutes* dominiert. Die Sequenzierung von DGGE-Banden zeigte eine Dominanz von *Firmicutes* in Zn-toleranten Kulturen. Aus Kulturen, denen Cu und Cd zugegeben war, wurde ein *Firmicutes*-verwandter Stamm isoliert. Diese Ergebnisse weisen auf die Bedeutung von *Firmicutes* für die Fe(III)-Reduktion in metallkontaminierten Böden

hin. Mit qPCR wurden 10^6 *Geobacteraceae* Genomkopien pro g Boden nachgewiesen, was 0.02 bis 0.04% der gesamten mikrobiellen Gemeinschaft im Btlc-Horizont entspricht.

Das FeRB *A. cryptum* JF-5 konnte mit Hilfe der Raman-Spektroskopie identifiziert werden, obwohl die Intensität der Raman-Signale aufgrund der Störung durch den Speicherstoff Polyhydroxybutyrat (PHB) abnahm. Mit Raman-Spektroskopie konnte zwischen aerobem und anaerobem Metabolismus und zwischen dem Wachstum mit gelöstem Fe(III) und einer Fe(III)-Festphase als Elektronenakzeptor unterschieden werden. Dies deutet auf die Existenz verschiedener Wege des Elektonentransfers zu beiden Fe(III)-Phasen hin. Weiterhin war die Unterscheidung zwischen Zellen, die in Gegenwart verschiedener Schwermetalle und unterschiedlicher Schwermetall-Konzentration gewachsen waren, möglich.

Insgesamt könnten Fe(III)-reduzierende Gemeinschaften im Btlc-Horizont die Freisetzung von Metallen, einschließlich Uran, verursachen. Diese Metalle könnten in tiefere Bodenhorizonte transportiert und im Br2-Horizont festgelegt werden. In der Zukunft könnten nach einer Zunahme des Kohlenstoffgehaltes reduzierende Prozesse auch auf der Gessenwiese einsetzen. Durch die Freisetzung von Metallen während der Fe(III)-Reduktion würde diese Fläche dann auch weiterhin eine Kontaminations-Quelle bleiben. Allerdings wurde in dieser Arbeit nicht untersucht, wie ein möglicher zyklischer Wechsel zwischen Fe(III)-Reduktion und Fe(II)-Oxidation den Rückhalt von Metallen beeinflussen könnte. Weiterhin werden die mikrobielle Fe(III)-Reduktion und damit verbundene Bodenfunktionen wahrscheinlich bereits durch geringe Metallkontaminationen gestört und könnten daher von einem Mechanismus zur Verringerung der bioverfügbaren Metallkonzentration oder einer ausreichenden Kohlenstoffquelle für den Entgiftungsstoffwechsel abhängig sein.

Eigenständigkeitserklärung

Ich versichere an Eides statt, dass ich die von mir vorgelegte Dissertation selbständig angefertigt und nur die von mir angegebenen Quellen und Hilfsmittel verwendet habe. Die Bestimmungen der Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena sind mir bekannt. Die Hilfe eines Promotionsberaters wurde nicht in Anspruch genommen und Dritte erhielten weder unmittelbar noch mittelbar geldwerte Leistungen, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen. Die Dissertation oder Teile davon wurde noch nicht als Prüfungsarbeit an der Friedrich-Schiller-Universität Jena oder an einer anderen Einrichtung für eine staatliche oder andere wissenschaftliche Begutachtung eingereicht.

Jena, den 25.03.2010

Ort, Datum	Eva-Maria Burkhardt

Hervorgegangene und geplante Publikationen

Alle Kapitel der Dissertation sind bzw. werden als Publikationen bei internationalen Fachzeitschriften eingereicht. Mein Beitrag an der Erstellung der vorliegenden sechs Manuskripte und Publikationen gestaltete sich wie folgt:

Küsel, K., **Burkhardt, E.-M.**, and Sitte, J. (2008) Effect of metal-reducing microorganisms on element fluxes in a former uranium-mining district. In: Liu, S.-J., Drake, H.L. (eds.) Microbes in the environment: Perspectives and challenges. *Science Press*, Beijing, 128-137.
Beprobung und Analyse des Porenwassers, sowie Durchführung von Mikrokosmen-Experimenten; die Arbeiten zum Gessenbach wurden in Zusammenarbeit mit Jana Sitte durchgeführt; Bestimmung der Gesamtzellzahl im glazialen Sediment der Gessenwiese; Bestimmung der Gesamtzellzahl und Ermittlung der An- und Abwesenheit bekannter FeRB-Gattungen im Uferboden des Gessenbachs mittels spezifischer PCR-Amplifikation des 16S rRNA-Gens durch Isabel Bauer; Erstellung des Manuskriptes durch K. Küsel.

Burkhardt, E.-M., Meißner, S., Merten, D., Büchel, G., Küsel, K. (2009) Heavy metal retention and microbial activities in geochemical barriers formed in glacial sediments subjacent to a former uranium mining leaching heap. *Chemie der Erde/Geochemistry* 69 S2: 21-34.
Erstellung des Manuskripts; Planung und Durchführung von Porenwasser-Analyse, Mikrokosmen-Vesuch, Gesamtzellzahl-Bestimmung und Auswertung der Daten; Anleitung von Sylvia Meißner bei der MPN-Analyse; Analyse der Boden-Festphase durch Sylvia Meißner, konzeptionelle Abstimmung und Planung der Arbeiten sowie Überarbeitung des Manuskriptes durch K. Küsel.

Burkhardt, E.-M., Akob, D.M., Bischoff, S., Sitte, J., Kostka, J.E., Banerjee, D., Scheinost, A.C., Küsel. K. (2010) Impact of biostimulated redox processes on metal dynamics in an iron-rich creek soil of a former uranium mining area. *Environmental Science and Technnology* 44: 177-183.
Erstellung des Manuskripts; Planung und Durchführung der Porenwasser-Analysen in Zusammenarbeit mit Jana Sitte; Planung und Durchführung des SIP-Mikrokosmen-Versuchs in Zusammenarbeit mit Jana Sitte und Denise Akob; Molekularbiologische Analysen des SIP-Versuchs in Zusammenarbeit mit Denise Akob; Anlegen der Klonbibliothek und Auswertung

der Daten; Durchführung des Metall-Dynamik-Vesuchs durch Sebastian Bischoff, XANES-Analysen wurden von Dipanjan Banerjee und Andreas Scheinost durchgeführt; konzeptionelle Abstimmung und Planung der Arbeiten sowie Überarbeitung des Manuskriptes durch K. Küsel.

Sitte, J., Akob, D.M., Kaufmann, C., Finster, K., Banerjee, D., **Burkhardt, E.-M.**, Kostka, J.E., Scheinost, A.C., Büchel, G., Küsel, K. Microbial links between sulfate reduction and metal retention in uranium- and heavy metal-contaminated soil. *Applied and Environmental Microbiology*, in press (23.03.2010).
Planung und Durchführung der Porenwasser-Analysen in Zusammenarbeit mit Jana Sitte; Planung und Durchführung des SIP-Mikrokosmen-Versuchs in Zusammenarbeit mit Jana Sitte und Denise Akob; Anmerkungenn zum Manuskript; Der Hauptteil der Arbeiten sowie das Erstellen des Manuskripts wurde von anderen Autoren ausgeführt.

Burkhardt, E.-M., Bischoff, S., Grube, S., Akob, D.M., Küsel, K. Metal tolerance of Fe(III)-reducing microbial communities in a contaminated creek soil. In preparation for *Applied and Environmental Microbiology*.
Erstellung des Manuskripts; Berechnung von Fe(III)-Reduktionsraten, DGGE und Sequenzierung der Banden; Kultivierung und Messung von FeRB mit Schwermetallen, DNA-Extraktion und Klonierung durch Sebastian Bischoff; Durchführung der qPCR durch Susanne Grube; MPN-Analyse sowie Isolierung von Mikroorganismen durch Denise Akob; konzeptionelle Abstimmung und Planung der Arbeiten mit K. Küsel.

Ciobotă, V., **Burkhardt, E.-M.**, Schumacher, W., Rösch, P., Küsel, K., Popp, J. The influence of intracellular storage material on bacterial identification by means of Raman spectroscopy. *Analytical and Bioanalytical Chemistry*, under revision (submitted 11.11.2009).
Planung der Versuche in Zusammenarbeit mit Valerian Ciobotă; Kultivierung von *Acidiphilium cryptum* JF-5, Beprobung und chemische Analytik der Kulturen, Bereitstellen von JF-5-Proben für die Raman-Spektroskopie; Die Raman-Spektroskopie und das Erstellen des Manuskriptes wurden durch Valerian Ciobotă und die anderen Co-Autoren durchgeführt.

Bestätigung des Eigenanteils an den Manuskripten:

Jena, den 24.03.2010

Ort, Datum	Kirsten Küsel

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